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THE INFLUENCE OF A SUSTAINED MONOCYTOSIS UPON THE ANTIBODY RESPONSE IN RABBITS TO VARIOUS ANTIGENS[1]

BY KENNETH F. GIRARD AND E. G. D. MURRAY

Abstract

By means of periodic injections of a chloroform soluble, nontoxic, and non-antigenic lipid substance extracted from *Listeria monocytogenes*, it was possible to stimulate and maintain a high degree of monocytosis in the blood of rabbits for at least twenty days. Animals immunized with *Salmonella typhosa*, staphylococcus toxoid, and horse serum during the period of monocytosis gave considerably higher titers than did control animals in every case.

Introduction

One of the peculiar characteristics of *Listeria monocytogenes* infection is the pronounced monocytosis and Stanley in 1949 (25) described the isolation and monocyte-stimulating properties of a lipid component of this organism. Using essentially the same methods we obtained a product which fitted the description given by Stanley and which, upon intravenous inoculation into rabbits, increased the number of circulating monocytes to a value approaching that produced in the active infection. Thus the *Listeria* lipid MPA (monocyte producing agent) seemed likely to provide a means to study the effect of a monocytosis upon the immune reaction in general.

Literature Review

Ever since the early experiments of Metchnikoff (21) the question of the relationship of antibody production to various kinds of cells in the animal organism has intrigued investigators. As successive new factors in immunity were demonstrated, the followers of Metchnikoff continued to ascribe the primary role to the system of macrophages and evidence of their participation in the antibody response was later obtained in "blockade" experiment with vital dyes and other colloids. These results have been extensively reviewed by Jaffe (17), and by Ehrich (8) who disagrees with that hypothesis.

Using a dye-antigen prepared by Heidelberger and Kendall, Sabin (23) found that the antigen was taken up principally by the cells of the reticulo-endothelial system and that coincident with the disappearance of the antigen

[1] *Manuscript received August 26, 1953.*
Contribution from the Department of Bacteriology and Immunology, McGill University, Montreal, Quebec.

in the cells and the appearance of antibody in the blood serum there was a marked shedding of the exoplasm of the macrophages. Her hypothesis was that the cytoplasmic "sheds" represented antibody globulin as well as normal globulin and that an antigen is a substance which can specifically modify the synthesis of cytoplasm components of the cells of the reticulo-endothelial system. The experiments of Manwaring (20) and Chase (4) working with the tubercle bacillus seemed to suggest an intracellular antibody phenomenon and the work of Lurie (18) supported such a suggestion. He observed that mononuclear phagocytes derived from animals immunized against tuberculosis and allowed to ingest tubercle bacilli *in vitro* while suspended in normal serum, when subsequently incubated in an environment devoid of immune body fluids, inhibited the multiplication of the microorganisms in their cytoplasm to a much greater degree than did mononuclear phagocytes from normal animals under the same conditions. He concluded that the active infection confers upon the macrophages bacteriostatic properties which are independent of immune body fluids. Goonder and Miller (13) reported that there was a correlation in their experiments between the numbers of monocytes present in the peritoneal cavity of mice at the time of infection and their resistance to pneumococcus Type 1 mixed with a suitable quantity of antiserum, but this is probably mainly a question of phagocytosis.

Studies by Stanley (26) showed definite correlation between the numbers of circulating monocytes and the antibody titrable *in vitro* against *Salmonella typhimurium*. Hartley (15) inoculated antigen (vaccinia virus) into macrophage nodules prepared by preliminary injection of aluminum hydroxide gel. Antibody was found in these areas before its appearance in the blood serum or other tissues and reached a higher titer. He claimed that concentration due to local inflammation was unlikely as antibodies did not reach as high a titer in such areas as they did in the blood serum when the antigen was injected intravenously. Walsh and Cannon (27) demonstrated an increased antibody content in the respiratory tissues of rabbits following the local application of vaccines to these tissues. They also showed that the tissues, so treated, exhibited an accumulation of monocytic cells in the subepithelial stroma. Walsh and Smith (28) found that 10 out of 15 rabbits with a macrophage-prepared pleural cavity, obtained by preliminary administration of a gum arabic – beef extract mixture, exhibited greater antibody response in the serum following intrapleural injection of vaccine than the unprepared control animals.

Attention was first drawn to the role of lymphocytes as regards antibody formation by McMaster and Hudack (19), later by Ehrich and his collaborators, and finally by White, Dougherty, and their colleagues. Their literature is too extensive to go into at this time and is adequately reviewed by Burnet and Fenner (3), but there is now a distinction drawn between the lymphocyte and the plasma cell ascribing antibody production to the latter. Several investigators have not been able to confirm the responsibility of a major role in antibody formation, transport, and release to the lymphocyte.

Craddock *et al.* (6) collected lymph and lymphocytes from the thoracic duct of cats immunized to a typhoid vaccine and failed to find antibodies in extracts from washed lymphocytes in spite of elevated titers in the cell-free lymph. Bjrneboe *et al.* (2), Habel *et al.* (14), Schweizer and Reber (24), and Erslev (9) similarly failed to find antibody in extracts of washed lymphocytes using quantitative chemical methods. Houghton *et al.* (16) failed to find that adrenal cortical hormones either increase the capacity of animals to form antibodies or alter titers after antibodies are formed. More recently, Fischel *et al.* (10) and De Vries (7) failed to find any rise in circulating antibody following administration of ACTH and cortisone to actively immunized animals, although marked lymphopenia occurred. Woodruff *et al.* (30) found that the administration of a potent antilymphocytic serum to rats immunized with *S. paratyphi B* failed to influence flagellar agglutinin titers despite a profound fall in the number of circulating lymphocytes. This fall in lymphocyte count occurred even in adrenalectomized animals, suggesting that it was not dependent on an adrenal mechanism.

There is therefore uncertainty of the importance of lymphocytes in antibody production. It is not our intention to go into the much-contested debate over the morphology of lymphocytes versus monocytes. The work of Witts and Webb (29) indicates that the cells appearing in the blood as a result of *Listeria* infection are correctly classified as monocytes. Conway (5), however, would consider them to be derived from lymphocytes. Toward this dilemma our attitude is that it is a physiological *in vivo* function with which we are concerned and not the morphological classification of the cells involved and by monocyte we mean those cells of the morphology described by Witts and Webb whose production is stimulated by *Listeria monocytogenes*.

Materials and Methods

L. monocytogenes was cultured in tryptose phosphate broth (Difco) by one of two methods. The method of Reed and McKercher (22) was employed at first, and the organisms grown for 24 to 48 hr. at 37° C. were collected from the cellophane surface into sterile distilled water, washed free of medium by centrifuging, resuspended in a minimal volume of a phosphate buffer (pH7.2), and then lyophilized in a Centrifugal Freeze-Dryer. In the second method, organisms were harvested by a De Laval Continuous Flow Separator from broth cultures in carboys in 20 liter quantities; the method is quick and very effective. With this method it was found expedient to aerate the culture during growth. Better utilization of the medium was apparent and the gm./liter relationship of organisms to medium was enhanced. The organisms were washed in the centrifuge and lyophilized as before. We also found membrane filters (15.0 cm., No. 5, Membranfilter-Gesellschaft, Sartorius-Werke, A. G. & Co., Gottingen, Germany) useful in collecting organisms from fluid medium on a large scale.

Chloroform used for extraction was purified before use by methods given by Bernhauer (1).

Experimental

An example of a typical extraction is the following: 19.54 gm. dry weight (lyophilized) of *L. monocytogenes*, representing about 50 liters of culture yielding 0.39 gm./liter, were pulverized with the aid of 4 mm. glass beads in a 200 ml. pyrex, glass-stoppered, flasks, by shaking on an electric vibrating machine in the presence of chloroform for about four hours, or until intact organisms could no longer be seen in stained smears of the bacterial debris. All of the products of cellular disintegration were then added to a separatory funnel containing 1500 ml. of chloroform placed in the cold room (5° C.) and shaken periodically for a week, after which the chloroform extract was separated from the bacterial debris by filtration through a membrane filter pretreated with the solvent. The chloroform extract was concentrated by distillation *in vacuo* at 37° C. The volume was then measured and the concentration of lipid/ml. was determined by evaporation of an aliquot over pure sulphuric acid *in vacuo*. Afterwards the concentrated extract was replaced in the distillation apparatus and sufficient 0.85% sodium chloride solution containing 0.03% sodium lauryl sulphate (or sodium desoxycholate) added to give a final concentration of lipid in the saline-detergent solution of about 30 mgm./ml. The distillation *in vacuo* was continued until the chloroform was removed. The concentration of the resulting dense suspension was measured by evaporation of an aliquot once more, making due allowance for the salt solution, and then adjusted by dilution to contain 20 mgm./ml. This was the stock suspension, labeled MPA and used to stimulate monocyte increases in experimental animals. In this case, the total lipid extracted represented about 5.12% of the dry weight of organisms. Biuret and other qualitative tests for protein were negative.

TABLE I

MONOCYTE NUMBERS MAINTAINED INCREASED OVER A 10 DAY PERIOD

Time in days	Injection of lipid MPA, mgm.	Total leucocytes	Monocytes, %	Monocytes, absolute numbers
0	30	10,250	3	307
1		12,500	8	1000
2		13,300	16	2128
3	20	13,000	22	2860
4		11,800	15	1770
5	20	15,400	13	2000
6		12,250	18	2210
7	20	13,100	16	2100
8		11,150	12	1338
9	20	13,500	20	2700
10		14,100	21	2835

In a previous report (11) we showed that intravenous inoculation of 20-30 mgm. of the *Listeria* lipid MPA caused a transient monocyte elevation; the monocytes rose to 2000 per cu. mm. in 48 hr. and began to diminish at 72 hr. until they reached a normal value at 120 hr. With this unquestionable rise in monocytes, the count of neither polymorphonuclears nor lymphocytes were significantly altered and morphologically the cells were indistinguishable from those occasioned by the actual infection. The test whether or not a monocytosis could be maintained at a high level over a sufficiently long period to study antibody responses, 30 mgm. MPA was injected intravenously in a rabbit and 72 hr. later, when monocytes had reached what would ordinarily be the peak value, another 20 mgm. was given and this dose was repeated every other day thereafter for a period of 10 days. Total and differential leucocyte counts were carried out every 24 hr. and the results are given in Table I, and Fig. 1.

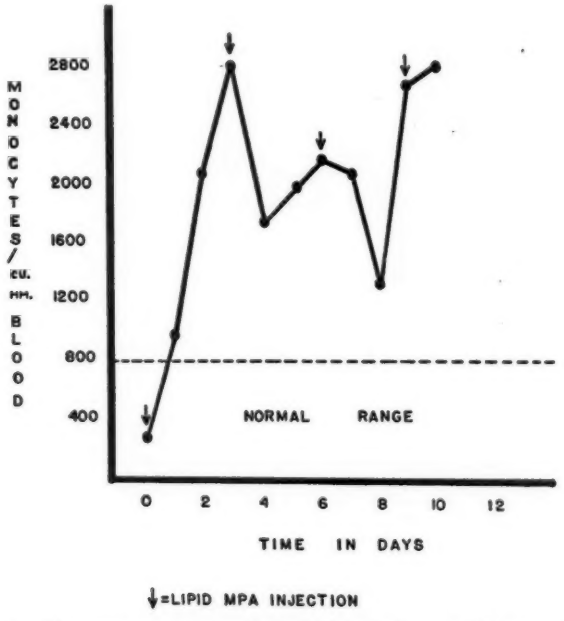

FIG. 1. Monocyte numbers maintained increased over a 10 day period.

In an experiment using two rabbits the test animal received periodic injections of MPA over a 20 day period, while both received *Salmonella typhosa* vaccine at intervals. Periodic bleedings were made, as well as total and differential leucocyte counts. The immunization schedule composed for this experiment is given below and the same pattern served for all subsequent experiments along this line. Results are shown in Table II.

IMMUNIZATION SCHEDULE

1st Day: Total and differential WBC; 30 mgm. of *Listeria* MPA (monocyte producing agent), intravenously.

3rd Day: 48 Hr. later, total and differential WBC; 0.1 mgm./kilo. of antigen(*S. typhosa*).*

4th Day: Antibody titration, 20 mgm. *Listeria* MPA intravenously.

6th Day: Total and differential WBC; antibody titration.

8th Day: 20 Mgm. MPA, 0.2 mgm./kilo. antigen, right and left ear vein respectively, total and differential WBC.

10th Day: Total and differential WBC; antibody titration.

11th Day: 20 Mgm. MPA.

13th Day: Total and differential WBC; 0.3 mgm./kilo. antigen intravenously; antibody titration.

15th Day: Total and differential WBC; 20 mgm. MPA.

17th Day: Total and differential WBC; antibody titration.

18th Day: 20 Mgm. MPA.

20th Day: Total and differential WBC; antibody titration.

** 0.1 Mgm. of S. typhosa is equivalent to about 500 × 10^6 organisms as determined with Brown's tubes.*

Summarizing: Injection of antigen every fifth day for a period of three weeks, and three injections.
Listeria MPA inoculations every 72 hr. (or fourth day).
Total and differential leucocyte counts, three times per week.
Titration of antibody, twice a week.

Control animals handled in identically the same manner without Listeria MPA injections.

TABLE II

RELATION OF ANTIBODY TO MONOCYTE NUMBERS OVER THE PERIOD OF IMMUNIZATION

Day	Antibody titrations				Absolute numbers, monocytes/cu. mm.	
	Test R. No. 1746		Control R. No. 1671			
	"O"	"H"	"O"	"H"	Test	Control
1st	−1 : 10	−1 : 10	−1 : 10	−1 : 10	186	246
3rd	—	—	—	—	2480	105
4th	−1 : 10	1 : 10	−1 : 10	−1 : 10	—	—
6th	1 : 20	1 : 40	−1 : 10	1 : 20	2260	592
8th	—	—	—	—	1500	930
10th	1 : 160	1 : 640	1 : 80	1 : 320	3025	360
11th	—	—	—	—	—	—
13th	1 : 160	1 : 1280	1 : 80	1 : 320	2750	334
15th	—	—	—	—	1300	300
17th	1 : 320	1 : 2560	1 : 160	1 : 640	2520	665
18th	—	—	—	—	—	—
20th	1 : 640	1 : 2560	1 : 320	1 : 1280	2850	256

Expt. No. 2: Antigen—S. typhosa "O" and "H".

It should be pointed out that the vaccine and lipid materials were not previously mixed but were injected separately and intravenously. Antibody titers show that the animal whose monocytes were increased, also showed a more rapid and more elevated response than the control. This result was encouraging and so the experiment was repeated employing five rabbits, three test animals and two controls, for each of three representative types of antigen: *S. typhosa* "O" and "H", staphylococcus toxoid, and horse serum; the immunization schedule as listed was followed. The composite experiments are given for each of these antigens in Tables III, IV, and V.

TABLE III

ANTIGEN, *S. typhosa*

Day	Rabbit No.	MPA, mgm.	Total WBC	% P	% L	% M	% E	% B	Absolute numbers, monocytes	Vaccine, mgm./kilo.	Antibody "H"	Titration "O"
1st	1770 T	30	11,800	45	40	5	5	5	590	—	—	—
	1771 T	30	10,100	22	70	5	2	1	505	—	—	—
	1772 T	30	9,400	49	43	3	2	3	282	—	—	—
	1773 C	—	13,500	45	50	2	1	2	270	—	—	—
	1774 C	—	10,300	28	70	1	1	—	103	—	—	—
3rd	1770 T	—	16,400	11	66	18	2	3	2950	0.1	—	—
	1771 T	—	13,450	15	63	22	—	—	2970	0.1	—	—
	1772 T	—	14,250	13	69	16	—	—	2280	0.1	—	—
	1773 C	—	9,500	30	65	2	—	3	190	0.1	—	—
	1774 C	—	8,200	32	63	3	—	2	246	0.1	—	—
4th	1770 T	20	—	—	—	—	—	—	—	—	−1:10	−1:10
	1771 T	20	—	—	—	—	—	—	—	—	−1:10	−1:10
	1772 T	20	—	—	—	—	—	—	—	—	1:20	−1:10
	1773 C	—	—	—	—	—	—	—	—	—	−1:10	−1:10
	1774 C	—	—	—	—	—	—	—	—	—	−1:10	−1:10
6th	1770 T	—	11,800	45	34	21	—	—	2475	—	1:10	−1:10
	1771 T	—	14,550	23	60	16	1	—	2320	—	1:40	−1:10
	1772 T	—	12,000	22	58	20	—	—	2400	—	1:20	−1:10
	1773 C	—	8,150	38	55	7	—	—	520	—	1:10	−1:10
	1774 C	—	5,350	25	70	4	—	1	214	—	1:10	−1:10
8th	1770 T	20	10,200	28	47	25	—	—	2550	0.2	—	—
	1771 T	20	12,800	20	60	20	—	—	2560	0.2	—	—
	1772 T	20	11,500	27	55	18	—	—	2065	0.2	—	—
	1773 C	—	9,800	15	82	3	—	—	294	0.2	—	—
	1774 C	—	10,000	32	65	3	—	—	300	0.2	—	—
10th	1770 T	—	15,250	37	42	21	—	—	3200	—	1:1280	1:20
	1771 T	—	11,700	38	44	18	—	—	2100	—	1:1280	1:40
	1772 T	—	14,250	22	60	18	—	—	2570	—	1:5120	1:80
	1773 C	—	10,500	15	80	5	—	—	550	—	1: 640	1:10
	1774 C	—	9,200	45	50	3	2	—	184	—	1: 640	1:20

Percentage leucocyte counts represent 200 cells enumerated and classified.
R No. 1774 expired on the 25th day, etiology unknown.

TABLE III—*(Concluded)*

ANTIGEN, *S. typhosa*

Day	Rabbit No.	MPA, mgm.	Total WBC	% P	% L	% M	% E	% B	Absolute numbers, monocytes	Vaccine, mgm./kilo.	Antibody "H"	Titration "O"
11th	1770 T	20	—	—	—	—	—	—	—	—	—	—
	1771 T	20	—	—	—	—	—	—	—	—	—	—
	1772 T	20	—	—	—	—	—	—	—	—	—	—
	1773 C	—	—	—	—	—	—	—	—	—	—	—
	1774 C	—	—	—	—	—	—	—	—	—	—	—
13th	1770 T	—	10,650	15	65	19	1	—	2030	0.3	1:12,800	1: 800
	1771 T	—	13,150	21	63	16	—	—	2100	0.3	1: 9,600	1: 800
	1772 T	—	14,800	18	66	16	—	—	2365	0.3	1: 6,400	1:1200
	1773 C	—	9,600	30	65	2	—	3	194	0.3	1: 3,200	1: 400
	1774 C	—	8,050	32	60	4	2	2	162	0.3	1: 3,200	1: 200
15th	1770 T	20	13,050	30	54	16	—	—	2100	—	—	—
	1771 T	20	13,150	28	56	16	—	—	2110	—	—	—
	1772 T	20	9,600	30	55	15	—	—	1440	—	—	—
	1773 C	—	10,000	32	66	2	—	—	200	—	—	—
	1774 C	—	8,500	24	72	3	1	—	85	—	—	—
17th	1770 T	—	11,200	46	32	22	—	—	2460	—	1:25,600	1:1200
	1771 T	—	13,700	32	60	18	—	—	2460	—	1:12,800	1:1200
	1772 T	—	10,500	40	48	12	—	2	1260	—	1:12,800	1:1200
	1773 C	—	6,750	32	62	4	2	—	270	—	1: 6,400	1: 800
	1774 C	—	9,700	25	70	3	—	2	291	—	1: 3,200	1: 400
18th	1770 T	20	—	—	—	—	—	—	—	—	—	—
	1771 T	20	—	—	—	—	—	—	—	—	—	—
	1772 T	20	—	—	—	—	—	—	—	—	—	—
	1773 C	—	—	—	—	—	—	—	—	—	—	—
	1774 C	—	—	—	—	—	—	—	—	—	—	—
20th	1770 T	—	14,800	35	45	20	—	—	2960	—	1:3200	1:400
	1771 T	—	13,450	27	50	23	—	—	3100	—	1:3200	1:400
	1772 T	—	13,550	31	55	14	—	3	1890	—	1:3200	1:400
	1773 C	—	9,200	28	69	3	—	—	276	—	1:3200	1:200
	1774 C	—	10,800	45	54	1	—	—	108	—	1:3200	1:200
		Weight, kgm.										
	1770 T	2.20										
	1771 T	2.34										
	1772 T	2.60										
	1773 C	2.49										
	1774 C	2.48										

Percentage leucocyte counts represent 200 cells enumerated and classified.
R No. 1774 expired on the 25th day, etiology unknown.

TABLE IV

STAPHLOCOCCUS TOXOID ANTIGEN

Day	Rabbit No.	MPA, mgm.	Total WBC	% P	% L	% M	% E	% B	Absolute numbers, monocytes	Vaccine, ml./kilo.	Antibody titration, P.I.U's/ml. serum
1st	1780 T	30	11,400	28	68	2	—	2	228	—	< 0.2
	1781 T	30	13,200	24	70	3	2	1	395	—	< 0.2
	1782 T	30	8,750	17	80	1	1	1	87	—	< 0.2
	1783 C	—	7,500	16	82	2	—	—	150	—	< 0.2
	1784 C	—	10,000	20	78	2	—	—	200	—	< 0.2
3rd	1780 T	—	15,500	25	60	15	—	—	2325	0.2	—
	1781 T	—	11,750	22	60	18	—	—	2110	0.2	—
	1782 T	—	15,600	35	45	20	—	—	3125	0.2	—
	1783 C	—	14,500	12	83	2	3	—	290	0.2	—
	1784 C	—	13,800	18	80	1	—	1	138	0.2	—
4th	1780 T	20	—	—	—	—	—	—	—	—	—
	1781 T	20	—	—	—	—	—	—	—	—	—
	1782 T	20	—	—	—	—	—	—	—	—	—
	1783 C	—	—	—	—	—	—	—	—	—	—
	1784 C	—	—	—	—	—	—	—	—	—	—
6th	1780 T	—	14,400	38	43	19	—	—	2740	—	< 0.2
	1781 T	—	13,500	42	36	21	—	1	2840	—	< 0.2
	1782 T	—	17,800	34	46	17	—	3	3050	—	< 0.2
	1783 C	—	13,550	56	40	2	—	2	270	—	< 0.2
	1784 C	—	9,700	36	60	2	—	2	194	—	< 0.2
8th	1780 T	20	13,855	37	45	18	—	—	2495	0.3	—
	1781 T	20	14,000	31	52	17	—	—	2375	0.3	—
	1782 T	20	15,150	35	43	22	—	—	3340	0.3	—
	1783 C	—	12,550	28	70	2	—	—	250	0.3	—
	1784 C	—	12,200	44	55	1	—	—	122	0.3	—
10th	1780 T	—	16,050	24	60	16	—	—	2570	—	—
	1781 T	—	12,750	29	45	23	1	2	2935	—	—
	1782 T	—	13,200	31	51	18	—	—	2375	—	—
	1783 C	—	9,050	27	66	3	4	—	362	—	—
	1784 C	—	13,300	21	74	4	1	—	133	—	—
11th	1780 T	20	—	—	—	—	—	—	—	—	12.8
	1781 T	20	—	—	—	—	—	—	—	—	6.4
	1782 T	20	—	—	—	—	—	—	—	—	6.4
	1783 C	—	—	—	—	—	—	—	—	—	3.2
	1784 C	—	—	—	—	—	—	—	—	—	3.2
13th	1780 T	—	14,100	28	52	20	—	—	2820	0.4	—
	1781 T	—	13,800	23	59	18	—	—	2480	0.4	—
	1782 T	—	15,200	29	50	21	—	—	3200	0.4	—
	1783 C	—	8,900	34	65	1	—	—	89	0.4	—
	1784 C	—	10,500	27	69	4	—	—	420	0.4	—
15th	1780 T	20	—	—	—	—	—	—	—	—	—
	1781 T	20	—	—	—	—	—	—	—	—	—
	1782 T	20	—	—	—	—	—	—	—	—	—
	1783 C	—	—	—	—	—	—	—	—	—	—
	1784 C	—	—	—	—	—	—	—	—	—	—

TABLE IV—*(Concluded)*

STAPHYLOCOCCUS TOXOID ANTIGEN

Day	Rabbit No.	MPA, mgm.	Total WBC	% P	% L	% M	% E	% B	Absolute numbers, monocytes	Vaccine, ml./kilo.	Antibody titration, P.I.U's/ml. serum
17th	1780 T	—	13,200	34	48	18	—	—	2375	—	25.6
	1781 T	—	14,450	29	53	18	—	—	2600	—	25.6
	1782 T	—	12,800	19	61	20	—	—	2560	—	12.8
	1783 C	—	10,500	31	67	2	—	—	210	—	6.4
	1784 C	—	12,000	36	60	4	—	—	500	—	6.4
18th	1780 T	20	—	—	—	—	—	—	—	—	—
	1781 T	20	—	—	—	—	—	—	—	—	—
	1782 T	20	—	—	—	—	—	—	—	—	—
	1783 C	—	—	—	—	—	—	—	—	—	—
	1784 C	—	—	—	—	—	—	—	—	—	—
20th	1780 T	—	14,800	28	53	19	—	—	2810	—	—
	1781 T	—	14,150	17	60	23	—	—	3250	—	—
	1782 T	—	13,200	30	45	25	—	—	3300	—	—
	1783 C	—	11,200	24	75	1	—	—	112	—	—
	1784 C	—	13,850	30	67	3	—	—	415	—	—
23rd	1780 T	—	—	—	—	—	—	—	—	—	12.8
	1781 T	—	—	—	—	—	—	—	—	—	12.8
	1782 T	—	—	—	—	—	—	—	—	—	6.4
	1783 C	—	—	—	—	—	—	—	—	—	3.2
	1784 C	—	—	—	—	—	—	—	—	—	3.2
		Weight, kgm.									
	1780 T	3.760									
	1781 T	3.255									
	1782 T	3.380									
	1783 C	3.640									
	1784 C	3.765									

Summary and Discussion

During periods of artificially induced hyperactivity of the monocyte-producing tissues, the antibody responses to injections of *S. typhosa* "O" and "H", staphylococcus toxoid, and horse serum were studied. Rabbits were divided into groups of five; in each group two rabbits were used as controls and the three test animals received periodic intravenous injections of the lipid MPA over the entire immunization period of 20 days. Total and differential leucocyte counts were carried out every 48 hr. on both control and test animals. Absolute monocyte counts of the test group ranged between 1500-3500/cu. mm., or about 17-25% of the total leucocyte count, and this is about three to six times the maximum normal. Monocyte counts on control animals rarely rose above 600/cu. mm. Blood was collected periodically for antibody determination and the immunizing doses were administered on the basis of mgm./kilo. body weight of rabbit. The three types of antigen all gave essentially the same results, that is, those animals

TABLE V

ANTIGEN, HORSE SERUM

Day	Rabbit No.	MPA, mgm.	Total WBC	% P	% L	% M	% E	% B	Absolute numbers, monocytes	Vaccine, ml./kilo.	Precipitin titer of serum
1st	1789 C	—	15,250	46	48	4	—	2	610	—	<1:5
	1790 C	—	9,300	17	80	3	—	—	280	—	<1:5
	1791 T	30	16,500	33	60	4	—	3	660	—	<1:5
	1792 T	30	11,500	30	67	3	—	—	315	—	<1:5
	1793 T	30	10,700	30	65	2	—	3	214	—	<1:5
3rd	1789 C	—	14,800	45	52	3	—	—	445	0.2	—
	1790 C	—	12,200	23	75	—	—	2	—	0.2	—
	1791 T	—	17,800	34	49	17	—	—	3030	0.2	—
	1792 T	—	14,650	14	70	16	—	—	2350	0.2	—
	1793 T	—	11,850	20	64	16	—	—	1890	0.2	—
4th	1789 C	—	—	—	—	—	—	—	—	—	—
	1790 C	—	—	—	—	—	—	—	—	—	—
	1791 T	20	—	—	—	—	—	—	—	—	—
	1792 T	20	—	—	—	—	—	—	—	—	—
	1793 T	20	—	—	—	—	—	—	—	—	—
6th	1789 C	—	8,850	24	72	4	—	—	355	—	<1:5
	1790 C	—	10,300	30	65	3	—	2	310	—	<1:5
	1791 T	—	12,800	25	53	20	—	2	2560	—	<1:5
	1792 T	—	14,200	31	48	18	—	3	2550	—	<1:5
	1793 T	—	15,200	25	55	16	2	2	2430	—	1:5
8th	1789 C	—	10,900	34	60	5	—	1	545	0.3	—
	1790 C	—	11,250	22	75	3	—	—	332	0.3	—
	1791 T	20	11,700	28	54	18	—	—	2100	0.3	—
	1792 T	20	15,100	31	52	17	—	—	2560	0.3	—
	1793 T	20	13,100	20	54	22	1	3	2880	0.3	—
10th	1789 C	—	8,750	25	72	2	1	—	87	—	—
	1790 C	—	10,000	42	55	3	—	—	300	—	—
	1791 T	—	11,050	28	55	15	—	2	1660	—	—
	1792 T	—	13,850	37	45	18	—	—	2500	—	—
	1793 T	—	12,250	35	39	20	3	3	2450	—	—
11th	1789 C	—	—	—	—	—	—	—	—	—	1:80
	1790 C	—	—	—	—	—	—	—	—	—	1:160
	1791 T	20	—	—	—	—	—	—	—	—	1:160
	1792 T	20	—	—	—	—	—	—	—	—	1:640
	1793 T	20	—	—	—	—	—	—	—	—	1:640
13th	1789 C	—	10,000	32	65	2	1	—	200	0.4	—
	1790 C	—	11,300	35	60	3	2	1	339	0.4	—
	1791 T	—	12,000	25	56	15	2	2	1800	0.4	—
	1792 T	—	14,850	31	52	17	—	—	2525	0.4	—
	1793 T	—	9,800	29	50	20	—	1	1960	0.4	—
15th	1789 C	—	9,500	24	70	4	1	1	380	—	—
	1790 C	—	11,000	25	65	5	2	3	550	—	—
	1791 T	20	14,500	32	49	16	2	1	2320	—	—
	1792 T	20	15,200	28	53	18	—	1	2740	—	—
	1793 T	20	13,350	24	58	18	—	—	2400	—	—

TABLE V—*(Concluded)*

ANTIGEN, HORSE SERUM

Day	Rabbit No.	MPA, mgm.	Total WBC	% P	% L	% M	% E	% B	Absolute numbers, monocytes	Vaccine, ml./kilo.	Precipitin titer of serum
17th	1789 C	—	10,500	29	68	3	—	—	315	—	—
	1790 C	—	12,200	24	72	2	2	—	149	—	—
	1791 T	—	14,250	23	50	22	—	—	3150	—	—
	1792 T	—	14,800	19	55	24	—	2	3552	—	—
	1793 T	—	13,850	30	48	21	1	—	2910	—	—
18th	1789 C	—	—	—	—	—	—	—	—	—	—
	1790 C	—	—	—	—	—	—	—	—	—	—
	1791 T	20	—	—	—	—	—	—	—	—	—
	1792 T	20	—	—	—	—	—	—	—	—	—
	1793 T	20	—	—	—	—	—	—	—	—	—
20th	1789 C	—	8,800	29	65	4	2	—	352	—	1: 320
	1790 C	—	8,850	36	57	4	2	1	354	—	1: 640
	1791 T	—	15,500	36	48	16	—	—	2480	—	1:1280
	1792 T	—	13,200	30	43	22	3	2	2900	—	1:2560
	1793 T	—	14,850	38	39	19	2	2	2820	—	1:1280

	Weight, kgm.
1789 C	2.580
1790 C	2.095
1791 T	2.770
1792 T	2.375
1793 T	2.770

whose monocytes were maintained increased showed increased antibody titers over those in the control groups. There was however, no strict parallelism between the absolute degree of monocytosis and the degree of immunity.

It should be emphasized that the lipid MPA was not previously mixed with the antigens, nor were the two ever inoculated by the same ear vein, thus the only factor to which we can refer our increased immunity responses in the test groups is to the monocytes which proliferated during the immunization periods. It is not asserted that the observed titer increases are necessarily attributable to the actual monocyte cells in the circulating blood, but it must be closely associated with processes involving the increase in these cells. It should be noted that the MPA itself is not antigenic and we have found it indifferently so combined with human globulins, when made to react as a Kahn antigen with positive syphilitic serum, injecting the collected flocculi intravenously into rabbits.

Perhaps, where facilities are available for X ray or other means of suppression of leucocytosis in test animals, while at the same time giving MPA and studying the immunity responses, the direct influence of MPA could be excluded. The method of Woodruff *et al.* (30) offers an approach likely to prove valuable. Obviously the histogenosis of these monocytes and their relation to the lymphocyte, plasma cell, and the reticulo-endothelial system

remains a matter of primary importance, especially in the light of the review of cellular sources of antibody by Ehrich (8). Our findings may necessitate a re-examination of the macrophage functions and this will be more evident from the results of extraction of monocytes to appear in another paper.

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THE PRESENCE OF ANTIBODY IN MACROPHAGE EXTRACTS[1]

By Kenneth F. Girard and E. G. D. Murray

Abstract

By means of intrapleural injections of gum arabic – beef extract mixtures into rabbits with an induced monocytosis it was possible to produce exudates wherein the predominating cell type was the monocyte. Exudates so-invoked in rabbits undergoing a secondary response showed that antibody was present in higher concentration within the exudate cells than in the supernatant fluid or blood plasma. However, passively introduced antiserum was also concentrated within these cells. Although it cannot be asserted that the monocytes themselves synthesize antibody, it nevertheless appears quite likely that these cells can take it up in considerable quantity.

Introduction

In a previous report (4) it was noted that when rabbits were immunized with various antigens during a period of sustained monocytosis, their immune responses were regularly enhanced. It therefore seemed possible that the monocyte might play some part in immune body synthesis. The following experiments were undertaken to determine whether homologous antibody was present in greater concentration in *extracts* of these cells, than in the suspending exudate fluid or blood plasma.

The practicability of such an approach was suggested to us by two reports: that of Walsh and Smith (8) who obtained large numbers of macrophage cells by intrapleural injection of rabbits with a gum arabic – beef extract mixture, and that of Erslev (2) who collected lymphocytes, washed and lysed them by sudden freezing – thawing, and examined the extracts for preformed antibody.

Materials and Methods

Handling of Exudates

These were collected from the pleural cavities of experimental animals. At first we used a 1% citrated-normal saline solution to collect the exudates and wash the cavities, but this only diluted the exudates still more and oftentimes small clots would form after standing. We next tried heparin but experienced similar difficulties. Finally 1 ml. of a 2% potassium oxalate solution was evaporated to dryness in small Erlenmeyer flasks (50 ml.) in a hot air oven at 120° C. for ½ hr. before use.

Preparation of Cell Extracts

(*A*) The whole exudate was centrifuged, washed once with Tyrode's solution, and the packed cells resuspended in 9% sodium chloride to a dilution of 1/10 by volume. The suspension was placed at 37° C. for two hours and

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Contribution from Department of Bacteriology and Immunology, McGill University, Montreal, Que.

then diluted 1 : 10 with distilled water, so that the final dilution of cells in the extract was 1 : 700 and the salt concentration 0.9%. This suspension was allowed to stand for two hours at 37° C. then centrifuged. Stained smears made of the sediment by the Wright and Giesma Methods usually showed only a few ghost cells, the remainder being unorganized sediment.

The method is a modification of that used by Murray (6) to disrupt Gram-negative bacteria. Essentially it consists of "soaking the cocci in strong brine until, by osmosis, a sufficient quantity of salt has penetrated the cells; then by the addition of distilled water, the concentration of salt in the fluid surrounding the cells is considerably reduced. As the water diffuses through the cell membrane more readily than the salt, a pressure develops within the cell which is sufficient to rupture the membrane". The method is, indeed, effective the only disadvantage being the necessity of diluting the extract to lyse the cells. Since our antibody titers were usually greater than 1 : 100, this was of no consequence.

(*B*) As an alternate method the cells could be lysed by immersion in a dry ice – absolute alcohol bath at −65° C. for a few moments, then in a bath at 37° C.; this was repeated five times.

Experimental

We felt that in the light of our previous findings (4), the increased numbers of monocytes could well be responsible for the enhanced immune responses observed but we had no way of collecting these monocytes for serological examination. The production of type-cell exudates was desirable and mixtures for this purpose were first tested in mice by intrapleural injection of 0.5 ml. of: (*a*) 40% gum arabic – MPA, equal parts; (*b*) 40% gum arabic – lecithin (20 mgm./ml.), equal parts; (*c*) liquid paraffin; (*d*) 40% gum arabic – 20% beef extract, equal parts.

MPA means monocyte producing agent, a chloroform-soluble lipid from *Listeria monocytogenes*.

In all four instances the predominating cell type after 24 hr. was the macrophage. Comparatively higher macrophage percentages were obtained with mixture (*d*) and so this material was used in the succeeding experiments.

Rabbits immunized three months earlier to *Salmonella typhosa* were used and their immunity was raised by a secondary response at the time of the experiment. The schedule of injections and collections was as follows: Rabbit No. 1772; day 1—intravenous injection of 30 mgm. MPA; day 3—intrapleural injection of 4500 × 10^6 dead *Salmonella typhosa* in 1 cc. to get a secondary response; day 5—intravenous injection of 20 mgm. MPA; day 7—intrapleural injection of 5–6 ml. equal parts 40% gum arabic in 20% beef extract; day 8—intravenous injection of 20 mgm. MPA; day 10—anesthetized with ether exsanguinated from the carotid artery and the pleural exudate collected. The results are given in Table I.

TABLE I

RABBIT 1772

S. typhosa titers		
Blood serum	— "H"	1 : 10,000
	"O"	1 : 1600
Supernatant exudate	— "H"	1 : 4000
	"O"	1 : 800
Exudate cell extract (osmotic method)	— "H"	1 : 10,000
	"O"	1 : 1600
Exudate character		
Total volume of exudate		18 ml.
Hematocrit		7% packed cells
Cell count		Rbc. 300,000/cu. mm.
		Wbc. 18,150/cu.mm.
Exudate differential count		Macrophage 70%
		Lymphocyte 20%
		Polymorphonuclear 10%

TABLE II

RABBITT 1773

S. typhosa titers "H"	
Blood serum	1 : 10,000
Supernatant exudate	1 : 5000
Exudate cell extract 1	1 : 6000
Exudate cell extract 2	1 : 5000 (smear of sediment here showed many more intact leucocytes than 1)
L. monocytogenes titers	
Blood serum	1 : 200
Supernatant exudate	1 : 800
Exudate cell extract 1	1 : 1600
Exudate cell extract 2	1 : 1600
Exudate character	
Total volume	22 ml.
Hematocrit	5% (Wintrobe Tube)
Rbc.	580,000/cu. mm.
Wbc.	14,150/cu.mm.
Differential	Monocytes.....75%
	Lymphocytes.....15%
	Polymorphonuclear.....10%

In another experiment the schedule employed was the same as shown in Table I except that on sixth day an intradermal injection of 5 ml. of *Listeria monocytogenes* antiserum of agglutinating titer 1 : 20,000 was given. The exudate was separated into two parts: (1) lysed by the osmotic pressure method; (2) lysed by sudden freezing–thawing with a dry ice–absolute alcohol mixture. The results are given in Table II.

Since the exudate cells were washed only once before being lysed, it seemed possible that antibody might be adsorbed on them. In order to evaluate this possibility as well as the presence of small amounts of blood in our exudates, whole blood (oxalated) taken at the same time from the same rabbit (1773) was treated in the identical manner, i.e. 0.5 ml. packed cells were lysed by the osmotic pressure method and the extract assayed. The results are given in Table III.

TABLE III

RABBIT 1773—CONTROL

Plasma vs. *S. typhosa* "H"	1 : 10,000
Saline washings of cells "H"	1 : 320 (equivalent volume to plasma removed)
Cell extract vs. *S. typhosa* "H"	<1 : 100
Plasma vs. *L. monocytogenes*	1 : 200
Cell extract vs. *L. monocytogenes*	<1 : 100

From the *S. typhosa* titers presented, especially with Rabbit 1773 in Table II, there seems very little difference between the exudate cell extract titer and supernatant exudate titer (1 : 6000 and 1 : 5000), but the following argument could indicate a marked concentration of *S. typhosa* antibody in the exudate cells. Judging from the control titrations in Table III, erythrocytes can be discounted as having anything to do with adsorption of antibody or otherwise containing it; hence the leucocytes of the exudates must have been responsible for the yield of antibody, and 75% of these were monocytes. Of the 0.5 ml. packed cells obtained by centrifuging half the exudate of Rabbit 1773, and discounting the inequality in size of leucocytes and erythrocytes, 2.36% by volume of the packed cells consists of leucocytes of which 75% are monocytes, giving a volume of 0.0089 ml. packed monocytes, so that the proportion by volume of monocytes to total volume of cells is 1 : 55. Thus in the original extract (undiluted) of the packed cells only one part in fifty-five parts would represent extract from macrophage-type cells. This represents the initial dilution of the intracellular antibody in the completed extract of cells of which the titration is shown in Table II. Using it as a correction figure, the intracellular titer of the typhoid antibody, produced by active immunity and related to the extract of monocytes in the exudate, becomes 1 : 339,000 as compared with 1 : 10,000 in the blood serum and 1 : 5,000 in the exudate supernatant. This is an enormous increment in the regional distribution of antibody. Calculated in the same way for the results given in

Table I, the initial dilution correction figure is 1 : 25 so the corrected titer for the intracellular antibody becomes "H" 1 : 250,000 and "O" 1 : 40,000. A passage of plasma into the chest cavity proportional to the erythrocytes in the collected exudate constitutes an extraneous source of antibody and further widens the gap between intracellular and extracellular antibody found in the exudate. The numbers of lymphocytes and neutrophyl leucocytes are too small to significantly detract from the preponderating monocytes as the source of intracellular antibody.

It seems evident from the results in Table II that there is a high concentration of passively introduced antibody in the exudate cells, since the exudate cell extract titer against *L. monocytogenes* antigen is also higher than the blood plasma or exudate supernatant fluid titers. Thus it cannot be argued that the cell extract *S. typhosa* antibody was formed by the cells in which it was found, for it could have been produced elsewhere and subsequently concentrated in the monocytes.

It was indeed surprising to find passively introduced antibody present in the intrapleural exudate to such an extent. We had anticipated only slight transudation. An experiment to verify this observation without the complication of active immunity seemed desirable. Rabbit 1793 was given 5 ml. of *L. monocytogenes* antiserum (1 : 20,000) intravenously and immediately thereafter the rabbit was given 5 ml. of the gum arabic–beef extract mixture, intrapleurally. After 48 hr. the animal was anesthetized lightly with ether, exsanguinated, and the exudate collected. The results of this experiment are given in Table IV.

TABLE IV

RABBIT 1793

Exudate character		
Exudate volume	5 ml.	
Volume of packed cells	0.75 ml.	
Rbc.	1,330,000/cu.mm.	
Wbc.	62,500/cu.mm.	
Differential	Monocytes	73%
	Lymphocytes	20%
	Polymorphonuclear	7%

L. monocytogenes agglutinating titers	
Supernatant exudate	1 : 1280
Cell washings	1 : 10
Blood serum	1 : 1280
Cell extracts	1 : 800

If we ignore once more the inequality in size of erythrocytes and leucocytes, the leucocytes represent 4.5% of the volume of massed cells collected and we calculate that the volume of packed leucocytes is 0.033 ml. and $0.75 \div 0.033 = 22.7$. Thus, in the original (undiluted) cell extract, leucocytes would be initially diluted at least 1/22. Using this as a correction figure, the intracellular titer becomes something of the order of 1 : 17,600 and compared to

1 : 1,280 for the blood serum and exudate supernatant it indicates a very considerable uptake of passive antibody by the cells. Because of the preponderating number of monocytes in the exudate we attribute this activity to them. Were we to use monocyte figures only, the dilution would be larger and the contrast between the intracellular titer and the titers of the blood serum and exudate supernatant would be greater still.

Discussion

The exudate cell extracts, when assayed for *S. typhosa* antibody produced by active immunity, showed higher titers than did the supernatant exudate fluid and the blood serum, indicating a greater concentration of antibody within these exudate cells. Had we not investigated the possibility of localization of passively introduced antibody in these cells it would have been easy to assume that the monocytes were the producers of the antibody in question. We did not prove or disprove that the monocytes were not immune body producing cells, but only that antibody could have been produced elsewhere and later concentrated within these cells. Nevertheless, it was indeed surprising to find preformed antibody taken up and liberated in the cell lysates to such a degree. These results conflict somewhat with those of Ehrich *et al.* (1) who assayed peritoneal exudate macrophage extract for antibody under somewhat the same circumstances as our own, and found the supernatant peritonal fluid to be about twenty times higher than the cell extract.

The impression is gained from these and past experiments that monocytes play a definite role in the immune response. That they can act as a storehouse for antibody has been previously suggested by the results of Lurie (5) and Garvey *et al.* (3), but our data also indicate that they may play a more important role particularly in those experiments wherein animals were immunized during a period of marked monocytosis (4). Whether, or not, immune globulin was actually produced within the cytoplasm of these cells, as Sabin (7) suggested, remains unanswered.

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THE PROTEOLYTIC ENZYMES OF MICROORGANISMS

VI. THE SEPARATION OF PROTEASES FROM *MORTIERELLA RENISPORA* DIXON-STEWART BY ZONE ELECTROPHORESIS[1]

By L. R. Wetter

Abstract

Moving-boundary electrophoresis indicated that a protease concentrate obtained from the culture medium of *Mortierella renispora* Dixon-Stewart (PRL 26) was made up of a number of proteins. Filter-paper electrophoresis demonstrated that two of the proteins were capable of hydrolyzing denatured hemoglobin. One active component had a negative mobility, the other a positive mobility in phosphate buffer pH 6.8, ionic strength 0.1. Because of the difference in electrical properties it was possible to separate the two active components by zone electrophoresis. Though yields were low when filter paper was employed, the use of potato starch as the supporting medium resulted in excellent recoveries.

Introduction

Since the partial purification of the extracellular proteolytic enzymes of *Mortierella renispora* Dixon-Stewart (PRL 26) was reported (9), further investigations have been performed regarding their electrophoretic properties. It is possible to ascertain the purity of an enzyme isolate by employing the Tiselius electrophoretic apparatus. If the sample is electrophoretically heterogeneous, one is then faced with the problem of determining which of the many components is the active principle. This becomes a difficult task when the standard Tiselius cell is employed especially if the enzyme has an intermediate mobility. The development of the filter-paper electrophoresis technique makes it possible to solve this problem quite simply.

Filter-paper electrophoresis was employed by Larson (5) and co-workers to demonstrate that radioactive iodine was concentrated mainly in the α_2-globulin of the serum. Nikkilä *et al.* (6) showed that the same technique could be used in enzyme studies. They were able to locate amylase and trypsin on a filter-paper strip by appropriate assay methods. Recently, Kunkel and Slater (2) have shown that potato starch provides a superior support as compared to paper for quantitative recoveries. The electrophoretic properties of oxytoxin (3) have been investigated by employing the paper technique.

This communication deals with the further purification of extracellular proteolytic enzymes by means of zone electrophoresis. A partially purified preparation was found to contain two components capable of digesting hemoglobin.

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Experimental

Methods

The moving-boundary electrophoresis experiments were done in the conventional manner using an Aminco Portable Electrophoresis instrument with a standard analytical cell.

Filter-paper electrophoresis was carried out as described by Kunkel and Tiselius (4), and Whatman (3 mm.) filter paper was employed. The paper strip was usually 37 cm. long but the width varied depending on the number of spots to be analyzed. The buffer-moistened paper was tightly clamped between two glass plates coated with a thin film of silicone grease, and the edges of the plate were then sealed with masking tape.* The electrode vessels were modified as follows: (*i*) carbon rod was employed as the electrodes rather than platinum, and (*ii*) a solid piece of plexiglas divided each electrode vessel and the two halves were connected by blotting paper. The paper electrophoresis experiments were performed at room temperature.

The protein on the paper strip was stained as described by Kunkel and Tiselius (4). The strip was oven-dried (100° C.), stained in the bromphenol blue – mercuric chloride – ethanol reagent and excess dye was removed by washing in 0.5% acetic acid. For a quantitative estimation, the stained strip was cut into 1 cm. segments and extracted in 0.01 *M* sodium hydroxide, the blue color was then read at 575 mμ in a Model B Beckman spectrophotometer. Later it was found that the protein content could be determined directly without elution. A "spotometer"** which could be used in a standard Model DU Beckman spectrophotometer was constructed according to Paladini and Leloir (7). Following the acetic acid wash, the damp dye-stained strip was exposed to ammonium hydroxide fumes. When the paper strip was completely dry it was placed in the "spotometer" and the color intensity read at 575 mμ. This method of protein assay was found to be quicker and readings could be taken at more frequent intervals than 1 cm.

Electroosmotic flow was determined by applying 0.005 ml. of 10% cellobiose at the origin. When the experiment was terminated the spot was developed by spraying the strip, after separating it from the main strip, with a phthalic acid – aniline reagent (8).

The proteolytic activity was determined by using urea-denatured hemoglobin as the substrate. After the completion of an electrophoretic run, the strip to be used for enzyme assay was cut into 1 cm. sections and each was extracted separately in 4 ml. of 0.9% sodium chloride for two hours. The enzyme content was determined on 1 ml. portions as described in a previous publication (9).

Early in the present investigation five or six layers of 3 mm. filter paper were used for the separation of the electrophoretic components. Since the yields were extremely poor when paper was employed, it was decided to use potato starch as the supporting medium. In the present work starch slabs

* *Canadian Minnesota Mining and Manufacturing Co., Ltd., London, Canada.*

** *Now sold by Fisher Scientific Co.*

0.5 cm. thick, 5 cm. wide, and 30 cm. long were used. The enzyme concentrate was pipetted into a "V-shaped" depression formed at the mid-point of the starch slab. The latter was wrapped in heavy waxed paper and then clamped between glass plates. After the completion of the experiment 1-cm. starch sections were extracted with 0.9% sodium chloride. The extract was tested for both protease activity and nitrogen content, the latter was determined by nesslerization (1).

Materials

The proteolytic enzymes were obtained from the fermentation medium of the mold *Mortierella renispora* Dixon-Stewart (PRL 26) as described previously (9). After reducing the volume by evaporation, the concentrate was freeze-dried and stored at 0° C. A solution of the dried material was fractionated once with 3.25 *M* ammonium sulphate, the precipitate was dissolved in water and dialyzed, the dialyzate was then freeze-dried. This sample served as the stock for all subsequent investigations.

Samples for paper and starch electrophoretic studies were prepared by dissolving 300 mgm. of the dried material in approximately 1 ml. of buffer. The enzyme solutions were then dialyzed against several changes of buffer. Finally the volumes were reduced to 1 ml. *in vacuo*. Aliquots of 0.02 to 0.04 ml. of these preparations were employed for electrophoretic analyses.

Results and Discussion

Moving-Boundary and Filter-paper Electrophoresis

One of the initial problems in which we were interested was the matter of purity of the ammonium sulphate precipitated fraction. The electrophoretic patterns (Fig. 1) showed this fraction to be quite heterogeneous. At least three components are clearly discernible and these have tentatively been designated as '1', '2', and '3'. The mobilities in phosphate buffer, pH 6.8

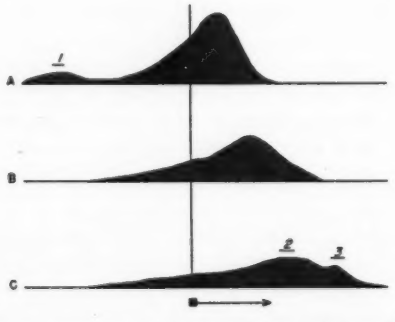

FIG. 1. Electrophoretic patterns of the ammonium sulphate precipitated fraction at ionic strength 0.1 and pH 6.8 (phosphate buffer). The potential gradient was 3.80 volts per cm. Exposures were taken at (A) 4355 sec., (B) 9,760 sec., and (C) 15,300 sec.

and ionic strength 0.1, are -5.8, $+1.2$, and $+1.9 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$ for '1', '2', and '3' respectively. Since spreading at the ϵ-boundary (initial boundary) is considerable, it becomes rather difficult to make an accurate estimate of the protein concentration. If the area of the ϵ-boundary is included the following relative composition is obtained: '1' 9%, ϵ-boundary 37%, '2' 38%, and '3' 16%. Component '1' appears to be quite homogeneous and for this reason it would be of interest to know whether it is enzymatically active. In addition since the pattern is complex, the question of which component is the protease becomes an important one.

To answer this problem the filter-paper electrophoresis technique was chosen for several reasons. First, the entire strip (or electrophoresis pattern) could be assayed for enzymatic activity. Second, the patterns are very similar to those obtained by the moving-boundary method (4) and therefore the two can be compared. Third, small amounts of material suffice for a complete assay, in the present study 1 mgm. of protein nitrogen was used for each spot.

The results of a typical filter-paper electrophoresis are shown in Fig. 2. The presence of two proteolytic active zones is clearly shown in 'A' of Fig. 2.

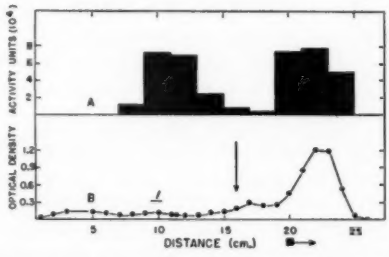

FIG. 2. Filter-paper electrophoresis pattern of the ammonium sulphate precipitated fraction at ionic strength 0.1 and pH 6.8 (phosphate buffer).
(A) A representation of the zones of activity.
(B) The electrophoretic pattern obtained by staining with bromophenol blue. The vertical arrow indicates point of application; horizontal arrow shows the position of the cellobiose spot after 18 hr. at 5 ma.

In addition, these two fractions appear to have approximately equal proteolytic activity. It is now possible to locate these enzymes on the electrophoretic pattern shown below in 'B'. Component '1' can be located in Fig. 2B and can be compared with Fig. 1. Since both these proteins have a negative mobility and are the only ones moving towards the anode there is no doubt that they are the same. Thus, it has been established that proteolytic activity is present in '1' as well as in the area made up of '2' and '3'. The component which appears just to the left of '1' in Fig. 2B is a concentration of brown material which gives the characteristic color to the fermentation. This material is not observed in Fig. 1 because it has passed out of the center section of the cell at the time the first exposure was taken.

The importance of using a reference spot such as cellobiose is well illustrated by Fig. 2B. It will be noticed that the carbohydrate has moved approximately 4 cm. towards the cathode because of electroosmosis. The position of the cellobiose spot after the completion of the run was, therefore, used as the initial boundary.

Fractionation by Zone Electrophoresis

The original method of fractionation by electrophoresis simply consisted of placing four or five sheets of Whatman (3 mm.) paper one on top of the other. The sample was applied to a flap which was cut through the paper layers and after applying the sample the flap was placed back into position (4). The electrophoresis experiment was carried out as described earlier. After the conclusion of the run, the damp papers were cut so that the two zones of activity were cleanly separated. The papers were then extracted with either distilled water or 0.9% sodium chloride. The yields were extremely poor and this was particularly true when distilled water was used as the extracting medium. Table I presents the results that were obtained. The cathodic component (this refers to the fraction moving towards the cathode) was isolated in extremely poor yield when extracted with distilled water. When saline was used the increase in yield was about fourfold for the cathodic component but insignificant for the anodic component.

TABLE I

PERCENTAGE EXTRACTION OF THE CATHODIC AND ANODIC COMPONENTS FROM WHATMAN FILTER PAPER (3 MM.) AND POTATO STARCH

Supporting medium	Extraction solvent	% of proteolytic activity extracted	
		Cathodic component	Anodic component
Paper	Distilled water	7	16
Paper	0.9% sodium chloride	28	19
Starch	0.9% sodium chloride	46	50

The following experiment was carried out to test the effect of water and saline on the extraction of the fractions from paper. Known quantities of the cathodic and anodic components were applied to squares (1 in.) of 3 mm. filter paper and then allowed to dry for 5 and 20 min. These squares were extracted either with distilled water or saline. Table II verifies previous results which suggest that the cathodic component is considerably more difficult to extract than the anodic one. Saline increases the amount removed from 6 to 10 times over that of water, whereas there is little difference for the anode fraction. Length of drying time also gives considerable differences. Probably this latter point plays a significant role.

TABLE II

A COMPARISON OF THE EXTRACTABILITY OF THE ANODE AND CATHODE FRACTIONS FROM FILTER PAPER (3 MM.) BY DISTILLED WATER AND SALINE (0.9% SODIUM CHLORIDE) FOR DIFFERENT PERIODS OF DRYING

Time (min.)	% of activity extracted			
	Cathodic component		Anodic component	
	Water	Saline	Water	Saline
5	9	96	72	82
20	7	46	44	34

Shortly after this investigation had been initiated, a report (2) appeared which stated that quantitative yields of proteins were obtained when potato starch was used as a supporting medium. The use of starch was therefore studied. An insoluble potato starch (Eimer and Amend) was employed and treated as described by Kunkel and Slater (2). When the electrophoretic run was completed, the starch supporting medium was cut into 1 cm. strips, extracted in saline, and assayed for both proteolytic activity and nitrogen content.

The typical electrophoretic pattern for semipurified protease was obtained (Fig. 3). A comparison of Figs. 2B and 3 illustrates that the two methods give the same result. This fact is particularly interesting when one considers that two different methods were employed to determine the protein concentration. In this study the bromphenol staining technique (Fig. 2B) is a good measure of protein when compared to the nitrogen content (Fig. 3). The proteolytic activity found in each segment is plotted in Fig. 3 and the agreement again is good between paper and starch. At the present time there is

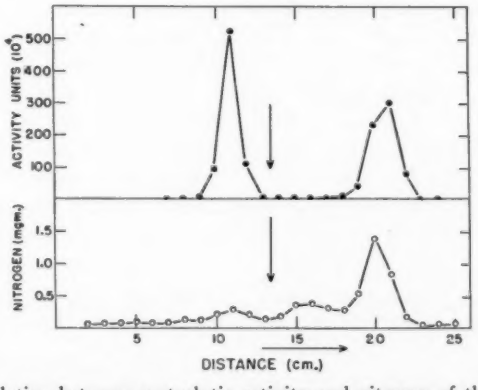

FIG. 3. Correlation between proteolytic activity and nitrogen of the electrophoresis of protease with the starch-supporting medium. Phosphate buffer ionic strength 0.1, pH 6.8. The vertical arrow indicates point of application.

no explanation of why single 1-cm. segments of filter paper result in almost quantitative yields while the batch method results in low yields. In the case of potato starch we obtained nearly quantitative recovery for both the activity and nitrogen, a matter of 96 and 100% respectively. Thus undoubtedly starch is much superior to paper in the matter of fractionation where yields are very often of prime importance.

The change in specific activity is of considerable interest. The original sample had a specific activity (units per mgm. of protein nitrogen) of 0.017. After fractionation on starch the values were 0.105 and 0.023 for the anodic cathodic components respectively. There are several ways of explaining this change, the anodic fraction is purer than the other fraction or it is a much more potent proteolytic enzyme.

Acknowledgment

The author is indebted to Miss J. Corrigal of this laboratory for her technical assistance in this study and to Mr. Spencer and the pilot plant staff who supplied the proteolytic enzymes used in this work.

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STUDIES ON THE ORAL TOXICITY OF *CLOSTRIDIUM BOTULINUM* TOXIN, TYPE A[1]

By Ivan W. Coleman[2]

Abstract

Three aspects of the reported oral toxicity of *Clostridium botulinum* toxin, Type A, were investigated. No demonstrable migration of the crystalline toxin from the lumen of the intestine into the blood stream of the dog could be found. Evidence indicating that the crystalline toxin was inactivated by pepsin and chymotrypsin was obtained, but the toxin was found to be resistant to the action of trypsin. Comparison of the oral toxicity and the intraperitoneal toxicity of the crystalline toxin revealed that the product was not orally toxic. A spray-dried crude preparation of the toxin demonstrated a low oral toxicity.

Introduction

The toxin of *Clostridium botulinum* has been described by Pappenheimer in his review of the proteins of pathogenic bacteria (11) as the only bacterial toxin which remains active when administered orally. Diphtheria and tetanus toxins, the only toxins of comparable toxicity, are both inactive orally, being rapidly destroyed by the proteolytic enzymes of the gut. Botulinum toxin is described as remaining toxic after *in vitro* treatment with pepsin and trypsin.

The toxin of *C. botulinum* has been crystallized by Abrams *et al.* (1) and Lamanna *et al.* (8). The type A toxin has been shown by Putnam *et al.* (15) and Kegeles (7) to be a protein of molecular weight 900,000 as determined by sedimentation and diffusion experiments. It is homogeneous in the ultracentrifuge at most pH values below pH 3 (19) and shows but one zone of migration in the electrophoresis apparatus over a pH range from 3 to 7 (14). The isoelectric point was shown (9) to be at pH 5.6. Elemental and amino acid composition of the toxin by Putnam *et al.* (15) revealed a protein of nitrogen value 16.29%. On the basis of a molecular weight of 900,000, this group advanced the amino acid residues as follows, according to the convention suggested by Brand and Kassell (2); $Gly._{166}$, $Ala._{394}$, $Val._{406}$, $Leu._{708}$, $Isoleu._{820}$, $Prol._{203}$, $Phen._{64}$, $CySH._{20}$, $(CyS–)_{40}$, $Met._{64}$, $Trp._{82}$, $Arg._{239}$, $His._{60}$, $Lys._{477}$, $Asp.NH_{1370}$, $Glut._{953}$, $Ser._{374}$, $Thr._{642}$, $Tyr._{672}$. This composition accounts for 100.2% of the total nitrogen.

The toxicity of the material is phenomenally high; per milligram of nitrogen it contains 1,200,000 toxic doses (minimum lethal dose per kilogram of animal) for a guinea pig, and 620,000 toxic doses for a mouse, on intraperitoneal injection (17). It is stable only at acid pH values, and is rapidly inactivated in neutral or alkaline solution.

It is difficult to reconcile the known physical properties of the toxin with the reported oral toxicity. The toxin is one which inhibits conduction at the

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myoneural junction (5, 18), death usually resulting from paralysis of the respiratory muscles. This requires that the toxin be absorbed from the intestinal lumen into the blood stream or lymphatic system, a process which would necessitate a protein of molecular weight 900,000 migrating across the gut membranes. It is difficult to imagine how a protein with such a molecular weight could penetrate the membranes of the gastrointestinal tract which are impermeable to proteins of less molecular weight. Dent (6) has reported evidence that dog serum proteins can be so absorbed, but these were restricted to serum proteins of the same dog. Part I of this report consists of an attempt to demonstrate the ability of the toxin to migrate across the gut membranes.

The amino acid constituents of the toxin do not supply evidence which would indicate a lack of the amino acids requisite to the formation of peptide linkages specifically opened by the action of the proteolytic enzymes, pepsin, trypsin, and chymotrypsin. A study of the action of the enzymes on the crystalline toxin comprises Part II.

In Part III a comparative study of the intraperitoneal and oral toxicities of the crystalline toxin and a crude preparation on mice and rats is reported.

Part I

Migration of *C. botulinum* Toxin Across the Gut Membrane

Materials

A sample of crystalline *C. botulinum* toxin, Type A, which was electrophoretically homogeneous, was available. The preparation in gelatine–phosphate diluent (1, 8) was found to have a minimum lethal dose by intraperitoneal route of 0.0001 μgm. per 20 ± 2 gm. mouse.

Method

Dogs 8 to 15 kgm. in weight were anesthetized with nembutal (40 mgm./kgm.). A loop of ileum was isolated through a small abdominal incision, and approximately 15 cm. tied off with two loops of surgical silk, leaving the circulation intact. One hundred micrograms of crystalline toxin in citrate buffer at pH 6.0 was introduced into the lumen of the intestine in one milliliter with a No. 22 needle, using a No. 18 needle as a trocar. The incision was then closed, and venous blood samples of 5 to 10 ml. in volume withdrawn under heparin at half-hour intervals for two and one-half hours. At the end of this period, 1 ml. of toxin containing 100 μgm. was injected intravenously. The animal was allowed to rest overnight without food or water. A terminal blood sample was taken 24 hr. after the first injection, before the animal was sacrificed. Plasma was separated from the blood cells and the toxicity tested by injecting 0.5 ml. into each of four mice. Deaths among these mice, 96 hr. after the injection, are recorded in Table I.

TABLE I

ABSORPTION OF *C. botulinum* TOXIN FROM ISOLATED SEGMENT OF GUT

Time of sample, hr.	Deaths among mice injected with 0.5 ml. plasma from:			
	Dog No. 1 14.5 kgm., male	Dog No. 2 14.1 kgm., female	Dog No. 3 15.4 kgm., female	Dog No. 4 8.7 kgm., female
0*	0/4	0/4	0/4	0/4
½	0/4	0/4	0/4	0/4
1	0/4	0/4	0/4	0/4
1½	0/4	0/4	1/4	1/4
2½	0/4	0/4	0/4	0/4***
20**	4/4	4/4	4/4†	3/4

* *Control sample before introduction of toxin into lumen.*

** *Time of intravenous injection of 100 μgm. of toxin in citrate pH 6.0.*

*** *Gut lumen was washed out with 2.0 ml. of gelatin phosphate solution which was tested for toxicity with four mice. Two mice died.*

† *0.5 ml. of filtered urine was injected into each of four mice, with no deaths resulting in 96 hr.*

The results indicate that, in the blood samples examined, only two deaths occurred in a period of two and one-half hours after the introduction of 100 μgm. of crystalline toxin into the gut lumen. However, after the intravenous injection of the same quantity, deaths occurred even when the blood samples were taken 20 hr. after the injection.

Discussion

In the dogs examined with the previously described technique, no evidence could be found indicating the migration of the toxin in an active form into the blood stream. Injection of the toxin intravenously indicated in every case the presence of the toxin in the plasma as long as 20 hr. after the time of injection. The parenteral dose applied was of the order of 200,000 mouse lethal doses, which, if 8% of the dog's body weight is blood (1.2 to 1.5 liters), would mean that an absorption of as little as 0.3 μgm. of the toxin is detectable. This represents an amount absorbed from the isolated gut lumen of less than 0.5% of the toxin introduced. Since conditions were such that a high concentration was maintained in the isolated lumen, it seems reasonable to expect that absorption would be of a higher order than 0.5% in two and one-half hours.

This argument takes no cognizance of the *in vivo* behavior of the toxin. Because no information exists on (*a*) the rate of tissue removal of the toxin from the blood stream, (*b*) the rate or extent to which the toxin can be absorbed on cell surfaces, (*c*) the reactions of detoxification possibly occurring in liver, kidney, or other body sites, no allowance can be made for that fraction of absorbed toxin which is by any mechanism removed from the plasma and hence not available for analysis by this method. It seems likely, however,

that the necessary correction for such reactions is small, since no difficulty was found in detecting the toxin in plasma 20 hr. after intravenous injection, but the rate of such reactions may still be such that something more than 0.5% of the toxin could have been absorbed from the gut lumen without detection.

Part II

The Digestion of *C. botulinum* Toxin by Pepsin, Trypsin, and Chymotrypsin

Methods

Buffered substrates of crystalline *C. botulinum* toxin, Type *A*, were prepared at 1 mgm./ml. concentration using 0.01 *M* potassium chloride – hydrochloric acid buffer at a pH of 1.4 for pepsin digestion, and 0.01 *M* phosphate buffer at pH 6.5 for trypsin and chymotrypsin. To 15 ml. of each substrate, 5 ml. solution containing 1 mgm./ml. of Armour crystalline pepsin, trypsin, and chymotrypsin was added. One 15 ml. portion of toxin substrate at pH 1.4 was retained as a control for the pepsin digestion, while a similar volume at pH 6.5 was retained as the undigested control for the trypsin and chymotrypsin digests. Five milliliters of water was added to each of the controls to maintain equivalent toxin concentration. Digestion was performed at 37° C. and allowed to proceed for 72 hr. One milliliter samples were withdrawn at regular intervals, and the toxicity determined by injections of 0.5 ml. samples intraperitoneally into mice. By serially diluting the digests sample (1:10 dilution at each step), the number of mouse lethal doses per milliliter of digest was determined. Groups of four mice were used at each dilution. They were arranged in weight groups: 20 ± 2, 25 ± 2, and 30 ± 2 gm. A minimum of 24 mice was used for each estimation of the toxicity of a digest sample. This required, with the controls, that 120 mice be used at each sampling time. Since groups of mice of the same weight in this number were not available, the three weight groups were chosen. The number of mouse lethal doses per milliliter of digest is therefore reported per kilogram of mouse.

Results

The toxicity of the crystalline pepsin, trypsin, and chymotrypsin preparations used was determined by injection of 0.5 ml. intraperitoneally into mice in graded concentrations to a maximum of 1.0 mgm./ml. No deaths resulted in any of the mice tested, even at the highest concentrations used. This step was taken to ensure that deaths resulting from the assay of the toxin digests could be ascribed to the toxicity of the toxin alone.

The results of the analysis of the digests and contents are shown in Fig. 1, in which the number of lethal mouse doses per kgm./ml. of digest is plotted against the time of digestion.

The results indicate that the toxicity of the controls decreases to approximately one-tenth of the starting value in 72 hr. This is in agreement with the findings of Reed and Muench (16).

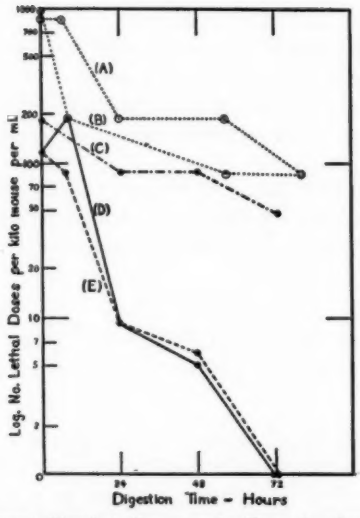

FIG. 1. Toxicity of crystalline botulinum toxin, Type A, after digestion with proteolytic enzymes: Curve A, control pH 1.5; Curve B, control pH 6.5; Curve C, trypsin digest: Curve D, chymotrypsin digest; Curve E, pepsin digest.

During the same period, the toxicity of the pepsin and chymotrypsin digests decreased rapidly after a short lag, to a value at 72 hr. at which the digests were nontoxic (at 1.0 mgm. toxin per ml.). The fugitive increase in toxicity of the chymotrypsin digest from zero to six hours may be significant. A similar process has been described by Bridgeman (3), Peterman (12), and Peterman and Pappenheimer (13), on enzymatic digestion of antibody molecules. These authors report that digestion of antibody molecules may proceed to the point of yielding residues of one-fourth the molecular weight of the original antibody without loss of the specific antibody activity. The transitory increase in the toxicity of the chymotrypsin digest may reflect such a process.

The action of trypsin was not as striking. At the end of the digest period the toxicity of the trypsin digest was about one-fourth the toxicity of the control at the same time. This may indeed represent the inertness of the toxin to trypsin digestion. However, since the terminal value of the toxicity is below the control, trypsin certainly exerts some action on the toxin. The lack of complete inactivation by trypsin may well be due to the inability to operate the experiment at or near the optimal pH trypsin digestion. The value of pH 6.5 used was necessitated by the rapid inactivation of the toxin at pH values more alkaline than 7.0. The digestion of the toxin at pH 8.0 to 8.4, the optimum pH of trypsin, would proceed more rapidly than shown here.

The uniform decrease in toxicity of the digests in comparison with the controls at zero time is probably due to the time interval between the withdrawal of the sample and the performance of the assay. The interval was of the order of one hour.

Summary

Pepsin and chymotrypsin digests of crystalline botulinum toxin, Type A, at pH values of 1.4 and 6.5 respectively, resulted in the inactivation of the toxin, such that the injection into mice of a volume of digest equivalent to that containing 1.0 mgm. of toxin (approximately 10^4 lethal mouse doses) had no effect.

Trypsin digestion did not result in complete inactivation when digested at pH 6.5, but in 72 hr. the digest showed a toxicity of approximately one-quarter of the similarly treated, but undigested, control.

Part III

The Comparison of Oral and Intraperitoneal Toxicities of Crystalline Botulinum Toxin Type A

Methods

A fresh solution of Type A crystalline botulinum toxin was prepared in citrate buffer pH 6.0. Several dilutions of the stock were made in gelatine phosphate diluent to yield six solutions ranging in concentration from 10 to 0.001 μgm. of toxin per ml. of solution. One milliliter of each dose was injected intraperitoneally into four mice in each group. For oral administration, 0.2 ml. was administered by stomach tube. The tube consisted of a flexible, 2-in. No. 26 needle, ball-tipped with silver solder. Ten animals were used in each oral dose group. The mice were kept in separate cages without food for the period of 48 hr., and the survivors were fed on regular rations until the conclusion of the assay period of 96 hr. Mice used were all females of 20 ± 2 gm. in weight.

The results of this comparison are shown in Table II, Section I.

The method previously described was repeated, using rats of 110 to 130 gm. in weight. The maximum oral dosage was increased to 1000 μgm. The results are also shown in Table II, Section II.

Spray-dried preparations of botulinum toxin, which had been prepared by precipitating the cell-free cultures by adjusting to pH 5.6, were available. The samples had been stored for three to four years, and still showed an LD_{50} of 0.002 μgm. per 20-gm. mouse by intraperitoneal route. Studies of the oral and intraperitoneal toxicity of this product were repeated on the rat in the manner previously described. The maximum oral dose of this crude preparation was increased to somewhat less than 7000 μgm. The results are shown in Table II, Section III.

Results

With crystalline toxin, application by the oral route of a dose which exceeded 2×10^4 times the minimum lethal intraperitoneal dose failed to cause any deaths in the mice tested. With the rat, increase of the oral dose to a value exceeding 10^6 times the minimum lethal dose intraperitoneally failed to show any effect on the rats tested. There is reason to believe that, with the crystalline

TABLE II

RESULTS OF THE ORAL AND INTRAPERITONEAL ADMINISTRATION OF BOTULINUM TOXIN TYPE A

Section 1						Section 2						Section 3					
Crystalline product Mouse 20, 2 gm.						Crystalline product Rat 120,10 gm.						Crude product Rat 120, 10 gm.					
Oral			Intraperitoneal			Oral			Intraperitoneal			Oral			Intraperitoneal		
Deaths per group	Animals per group	Dose, μgm.	Dose, μgm.	Animals per group	Deaths per group	Deaths per group	Animals per group	Dose, μgm.	Dose, μgm.	Animals per group	Deaths per group	Deaths per group	Animals per group	Dose, μgm.	Dose, μgm.	Animals per group	Deaths per group
0	10	0*	0	4	0	0	10	0*	0*	10	0*	0	10	0*	0*	8	0
0	10	2.10	1.0	4	4	0	10	1000	1.0	8	8	10	10	6870	68.7	8	8
1	10	0.2	0.1	4	4	0	10	100	0.1	8	8	3	10	687	6.8	8	8
0	10	0.02	0.01	4	4	0	10	1.0	0.01	8	8	0	10	69	0.68	8	8
0	10	0.002	0.001	4	4	0	10	0.1	0.001	8	3	0	10	6.9	0.07	8	8
0	10	0.0002	0.0001	4	3	0	10	0.01	0.0001	8	0	0	10	0.069	0.007	8	8
												0	10	0.07	0.0007	8	1

* *Controls of gelatine–phosphate diluent.*

product, the oral dose could be raised still further without being toxic. With the crude preparation there was a demonstrable oral toxicity at a level which represented 10^6 times the minimal effective intraperitoneal dose.

Discussion

The lack of demonstrable oral toxicity of crystalline botulinum toxin, Type A, is in agreement with the results obtained in the permeability study and with the inactivation of the toxin by pepsin, chymotrypsin, and probably trypsin. It is, however, in sharp contrast with the results reported in the literature (10, 11) and others which described the toxin as being effective orally. The previous observations of the oral toxicity have been made by many authors and are well documented in the clinical cases of fatal botulism in which the oral route could be the only method of admission of the toxin. However, it must be remembered that all the observations have been made (to the best of the author's knowledge), on either intact cultures of *C. botulinum* or on cell-free filtrates. The toxicity of the crude products has then been ascribed wholly to the toxin. It is only this conclusion that is erroneous. If the hypothesis is advanced that in the process of isolation and purification of the crystalline toxin a second oral potentiating factor of unknown properties is lost, the results of this work and the previous reports can be reconciled. Evidence in favor of such an hypothesis has already been advanced by Bronfenbrenner and Schlesinger (4). These workers found that the addition of ammonium sulphate to cell-free cultures of *C. botulinum* reduced the oral toxicity of the culture to one hundredth of its previous value, although the intraperitoneal toxicity remained the same. On addition of the material precipitated by ammonium sulphate, the oral toxicity of the reconstituted media returned to its original value. The results obtained with crude spray-dried botulinum toxin tend to support this concept. The oral toxicity of this product can be explained by assuming that it still contains some of the postulated factor.

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INTRACELLULAR DISTRIBUTION OF DESOXYRIBONUCLEODEPOLYMERASE IN NORMAL RAT LIVER, LIVER TUMOR, AND LIVER OF ANIMALS FED *p*-DIMETHYLAMINOAZOBENZENE[1]

By Gaston de Lamirande[2], Claude Allard, and Antonio Cantero

Abstract

The intracellular distribution of desoxyribonucleodepolymerase (DNase) has been investigated in the liver of animals fed *p*-dimethylaminoazobenzene (DAB), in liver freed from tumor, and in DAB induced tumor. The method is based on the determination of acid soluble material containing phosphorus, liberated by the action of the enzyme upon highly polymerized DNA. Results indicated that the nuclear DNase particularly accounts for a very low percentage of the whole cell activity in normal rat liver, whereas in nuclei of liver of DAB fed rats and of tumor the activity is increased to a high level. These facts suggest a possible correlation between the activity of DNase in the nuclear fraction and the mitotic rate of the tissue.

Introduction

Variations in desoxyribonucleodepolymerase (DNase) activity have been previously reported in the liver of rats fed *p*-dimethylaminoazobenzene (DAB) (4). An increase was noted in DNase activity associated with early damage caused by DAB, which is followed by a return to normal level during the ensuing regeneration and tumor formation resulting from prolonged action of the carcinogen. The possibility that this early and transitory variation of the DNase activity might be characteristic of liver carcinogenesis prompted us to resume the study of this enzyme at the intracellular level.

Methods

Wistar white rats of an average body weight of 200 gm. were fed a semisynthetic diet in which DAB was incorporated at a concentration of 0.06%. Control animals were fed the semisynthetic diet alone (8, diet 3). The animals were killed by decapitation, the livers were excised, homogenized in 0.25 *M* sucrose, and centrifuged to obtain three cellular fractions. The fractions were made up at the following concentrations as compared to the 10% original homogenate (100 mgm. per ml.); nuclear and mitochondrial fractions, 20% (200 mgm. per ml.); supernatant fraction which contained the nonsedimentable part of the cell, 4%. This has been fully described in a previous paper (2).

As in previous work (1), the tumors were obtained from rats fed the DAB diet for 150 consecutive days followed by a period of two months on a normal diet (Purina Fox Chow). Only small tumors of an average diameter of 1 cm. were chosen. Necrotic tissue was eliminated as much as possible. The

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[2] *Fellow of the Damon Runyon Memorial Fund for Cancer Research Inc.*

remaining liver was cleared of visible tumor tissue and designated as liver freed from tumor. Tumor tissues as well as liver freed from tumor were treated as described above. The number of cells and mitochondria per gram of fresh tissue were determined by actual counts on the homogenate and cell fractions aliquots. Corrections for the presence of mitochondria in the nuclear fractions were made as previously described (2). The tumors were not studied histologically.

The DNase activity of the homogenate and of the cell fractions was measured by the modified method of Laskowski (7). The experimental tubes contained 1.5 ml. of 0.6% DNA* solution, 0.5 ml. of 1 *M* acetate buffer pH 5.0 and 0.5 ml. of homogenate of tissue fraction. The tubes were incubated for three hours at 37° C. and the reaction stopped by adding ice-cold 10% trichloroacetic acid. The acid soluble fraction was then digested with concentrated sulphuric acid and the phosphorus content determined by the method of Fiske and Subbarow (5). Appropriate controls were carried out with each series of experiment. The nitrogen determinations were done by micro-Kjeldahl method.

Results and Discussion

Table I shows the intracellular distribution of DNase activity in liver homogenates prepared from normal rats, rats fed control or DAB diet, and homogenates of liver tumor and liver freed from tumor. The results are given in percentage of the total homogenate activity and in γ of acid-soluble phosphorus liberated per gram of fresh tissue. Results are also given in γ of soluble phosphorus liberated per cell and per mgm. of nitrogen (specific activity). Values for the nuclear and mitochondrial activities in this table have been corrected for the actual number of mitochondria present in the nuclear fractions (2).

Percentage Distribution

Table I indicates that in normal liver cells, the cytoplasmic fractions (mitochondrial and supernatant) contain a high percentage (93.4) of the total homogenate activity. This high percentage of activity is about equally distributed between both fractions (44.4 and 49.0% respectively). Only 6.6% of the total activity is found in the nuclear fraction. Actual cell counts on the isolated nuclear fraction showed that about 5% of unbroken cells were present in this fraction owing to incomplete homogenization. The percentage of activity found in the nuclear fraction may thus be accounted for, to a large extent, by the presence of unbroken cells. This conclusion is further supported by the fact that pure nuclei, prepared by the modified method of Hogeboom and Schneider (6), were devoid of appreciable DNase activity (Table II). Nuclei of normal liver cells would thus possess a negligible DNase activity.

* *Commercial highly polymerized desoxyribonucleic acid (sodium salt) from Bios Laboratory.*

TABLE I

INTRACELLULAR DISTRIBUTION OF NUCLEODEPOLYMERASE ACTIVITY OF RAT LIVER IN NORMAL AND PATHOLOGICAL CONDITIONS

	Normal liver	90 days control	90 days DAB	Liver freed from tumor	Tumor
Number of animals.....	4	5	6	3	5
Number of cells/gm. $\times 10^6$	143	161	178	132	98
Number of mitochondria per cell	2440	2750	1778	3427	1816
Liver fractions	Percentage distribution				
H	100	100	100	100	100
N	6.6	7.5	19.6	16.2	18.1
M	44.4	56.0	51.5	42.7	26.2
S	49.2	41.7	33.7	36.0	56.0
Recovery	100.2	105.2	104.8	94.9	100.3
	γ of phosphorus liberated per gm. of tissue				
H	4948	5127	5981	6182	4602
N	328	385	1166	1000	807
M	2197	2871	3073	2632	1173
S	2435	2135	2011	2227	2580
	γ of phosphorus liberated per cell $\times 10^{-6}$				
H	34.6 ±10.6	32.1 ±10.4	33.6 ±12.0	46.7 ± 8.7	47.0 ±12.0
N	2.3 ± 1.6	2.3 ± 0.7	6.7 ± 3.6	7.6 ± 1.3	8.2 ± 3.7
M	15.3 ± 6.5	17.8 ± 7.1	16.9 ± 5.2	19.9 ± 3.3	12.0 ± 5.4
S	17.0 ± 8.4	13.3 ± 0.8	11.3 ± 4.8	16.9 ± 4.6	26.3 ±10.3
	γ of phosphorus liberated per mitochondrion $\times 10^{-10}$				
	72.0 ±20.0	65.0 ±10.2	77.8 ±17.8	65.1 ± 3.2	65.7 ± 5.0
	γ of phosphorus liberated per mgm. of nitrogen				
H	168.5	160.5	242.8	207.3	208.0
N	84.7	132.2	253.9	150.0	203.0
M	318.3	258.9	355.6	438.8	341.2
S	139.9	118.3	142.6	177.1	173.0

The distribution of DNase activity in livers of animals fed control diet for 90 days (Table I) is not significantly different from normal liver. This would indicate that a low protein diet, as used in this study, does not affect the total liver DNase activity nor its intracellular distribution. However, in liver of animals fed DAB for 90 days, in liver freed from tumor as well as in liver tumor, the distribution of DNase activity is different from that of normal or control livers. In fact, the most striking result is the high percentage of DNase activity present in the nuclear fraction. Such high percentages of nuclear activity, namely 19.9, 16.3, and 17.4% (after correction for the number of mitochondria present in these fractions) could not be accounted for by the presence of unbroken cells (5%). Moreover, preparations of pure nuclear fractions of liver tumor showed precentage activity of 14.5% (Table II). Nuclei of liver cells, as noted in the above conditions, would therefore seem to contain an important percentage of the total activity of the cell. Similar results have been obtained in this laboratory for acid β-glycerophosphatase.*

TABLE II

NUCLEODEPOLYMERASE ACTIVITY OF NORMAL AND TUMOR PURIFIED NUCLEAR FRACTION*

Number of assays	Normal		Tumor	
	%	Per cell	%	Per cell
1	0.0	0.0	14.5	6.8
2	1.1	0.4		

* *Nuclear fractions were prepared by the modified method of Hogeboom and Schneider* (6). *Actual counts revealed the presence of 0.5 to 1.0% mitochondria in these fractions.*

In the liver mitochondrial fraction of animals fed DAB for 90 days and of liver freed from tumor, the percentage distribution of DNase activity is similar to control and normal liver respectively. However, in DAB induced tumor, 25.5% of the total DNase activity is distributed in the mitochondrial fraction as compared to 44.4% in normal animals. A slight decrease, in the percentage distribution of the supernatant fraction, is observed in animals fed DAB and in liver freed from tumor as compared to control and normal liver, whereas an increase is observed in liver tumor.

Activity per Gram of Fresh Tissue and per Average Cell

The DAB feeding and the formation of tumor seems to affect the total DNase activity of the tissue when calculated on a per gram basis, since the level is higher by 14 and 20% respectively in animals fed the carcinogen for 90 days and in liver freed from tumor. However, in the tumor itself, the total activity

* *Unpublished data.*

is slightly decreased (8%). A somewhat different picture is obtained when the results are expressed per average cell. The increase in DAB fed rats is no longer evident, indicating that it is due to a greater number of cells rather than to a real increase of activity. However, the higher level observed in liver freed from tumor per gram of fresh tissue is more pronounced per cell (increase from 20 to 35%), whereas the slight drop in the tumor DNase activity per gram of fresh tissue is now observed to be an increase of 35% per cell. The increase in total DNase activity of the homogenate calculated on either basis of expression are mostly caused by the large increase noted in the nuclear fraction activity which is about three times that of normal and control animals.

The variation observed in the mitochondrial fraction activity, which is more marked in tumor (decrease of 47%), is less accentuated when the results are expressed in a per cell basis, and are nonexistent when the activity is expressed per mitochondrion. It would seem that these variations are due to a variation in the number of mitochondria rather than to a change in the mitochondrion itself.

Specific Activity (Activity per Mgm. of Nitrogen)

The total DNase specific activity of animals fed DAB for 90 days is increased by 50% as compared to controls. This confirms previous results (4). However this increase is due to a depletion of nitrogen per average cell, since a normal value for the enzyme activity is obtained when calculated per average cell.

Variations of the specific activity which may be noted in liver homogenates and liver cell fractions in the different conditions studied are different when calculated per average cell. This would indicate that variations of nitrogen and enzyme activity are not parallel and of the same extent in all the fractions.

Interpretation

The most significant finding of the present work appears to be the increased nuclear DNase activity which is associated with increases in mitotic rates in the liver of rats fed DAB for 90 days, in the liver freed from tumor, and liver tumors*. While several references exist in the literature for the DNase activity of whole homogenate of various tissues, only few attempts have been made as yet to estimate the DNase activity of various cell fractions. The facts reported that mouse liver nuclei and kidney nuclei do not contain DNase activity (9), and that thymus nuclei contains DNase activity (3), suggest that DNase activities of the nuclear fractions may be related to the mitotic activity of these tissues.

It has been shown recently that the DNA of the mother cell, which is highly stable in nondividing cells, is degraded and replaced by twice its

* *Quantitative data are being obtained, in this laboratory, concerning the mitotic rates of these tissues.*

amount of new DNA at the "duplication stage" of mitosis (11, 12). A correlation between nuclear DNase and incidence of mitosis would thus suggest that nuclear DNase is involved in such a renewal of the mother DNA taking place at mitosis. Further work would be required in order to establish whether such a correlation holds for other tissues.

The possibility that liver tumor, liver freed from tumor, and liver of rats fed DAB contain a population of cells not usually present in high proportion in normal liver (13), such as bile duct cells, cannot be excluded. These cells may possibly contain nuclear DNase which thus could be determined in certain pathological conditions. This view is in line with the interpretation of Schneider *et al.* who assumed a different intracellular distribution of DNase in parenchymal and nonparenchymal cells in liver of rats fed 3′-methyl-4-dimethylaminoazobenzene (10). Histochemical investigation of this problem as well as the study of numerous tissues with different mitotic activity would be necessary.

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STUDIES ON THE SUBACUTE AND CHRONIC ADMINISTRATION OF STILBESTROL IN THE MALE RAT[1]

By D. W. Snair, Sybil E. Jaffray, H. C. Grice, and L. I. Pugsley

Abstract

The temporary effects of the administration of stilbestrol upon body weight, weight of the accessory sex organs, and the reproductive capacity of the male white rat have been studied. A linear relationship was obtained when the logarithm of the dose of stilbestrol administered (0.0125 to 5.0 mgm.) was plotted against the percentage decrease in testes weight. This same relationship was also found when the log of the dose was plotted against the percentage decrease in body weight although this line had a much more gradual slope.

When the administration of stilbestrol was discontinued, the seminal vesicles coagulating glands, and ventral prostrates regained their weight by the third to fourth week but the weights of the testes remained below the control values until the sixth to ninth week. In a breeding experiment it was shown that a daily dose of 7.5 μgm. of stilbestrol causes sterility in the male. This does also slightly affected the weight of the accessory sex organs and their cellular structure.

Introduction

Since the discovery by Dodds and co-workers (2) of the estrogenic properties of 4, 4′-dihydroxy-α-β-diethylstilbene (stilbestrol) a number of investigators have reported the effect of the administration of this compound on body weight and the weight of the accessory sex organs of the male rat.

Morrell and Hart (14,15) and others (13,16,20) have reported that stilbestrol causes a retarding effect on body growth in young male rats and a loss of body weight in mature animals. These workers (14, 15) and others (4, 10, 17, 21) have also noted that this synthetic estrogen in small doses over varying lengths of time caused a suppression of the function of and a reduction in the weight of, the seminal vesicles, ventral prostate, and testes in the male.

Histological studies (11,14,15,21,26) have shown that large doses of stilbestrol cause marked degeneration in the seminiferous tubules of the testes in some animals, but in others damage may be slight with some normal sperm production.

Hoskins and Small (8) in tests on senescent male rats found that injections of stilbestrol consistently resulted in an increase in spontaneous activity of from 50–3000%. These same treatments produced a body weight loss of from 16–30%. The initial weight was regained in about three weeks after stilbestrol administration was discontinued.

Noble (16) has reported that atrophy of the gonads was not produced until a daily dose of approximately 7 μgm. was administered. This investigator has also shown that the atrophy of accessory sex organs produced in stilbestrol treated male rats is as severe as that produced by hypophysectomy (9).

[1] *Manuscript received August 31, 1953.*
Contribution from the Food and Drug Laboratory, Department of National Health and Welfare, Ottawa, Canada.

Matthews *et al.* (11) in studies on the effect of stilbestrol on the breeding capacity of the male have shown that an average period of 47.5 days was necessary for the testes to regain a functional state after the animal received 1 mgm. of stilbestrol three times weekly for a period of 56 days.

In view of the fact that stilbestrol has been recommended as a fattening agent in poultry and other animals used for food and since residues of this estrogen may possibly accumulate in the flesh of animals so treated, it appeared of interest to study more closely the effects of the administration of small daily doses of this estrogen to male rats over varying periods of time, with respect to body weight loss, accessory sex organ weight loss, histology of testes, recovery time, and breeding activity.

Methods

Albino rats of Wistar strain maintained on Masterfox cubes* and water ad libitum were used in these experiments. In any one experiment in which organs from a group of injected animals were compared with those of a control group the range of body weight within and between the groups was kept the same (± 10 gm.). The animals were killed with chloroform and the testes, seminal vesicles, coagulating glands, and ventral prostate were carefully removed, dissected free of extraneous tissue, and weighed to 0.2 mgm. on a Roller Smith torsion balance.

Experimental

In the first experiment three plans of injection were followed, each consisting of three dosage levels of stilbestrol, 75, 150, and 300 μgm. In the first plan these total doses were given in five daily injections. The animals were killed and the body and testes weighed on the sixth day. In the second plan the total dose was given in 10 daily injections and the animals were killed on the 12th day. In the third plan the total dose was given in 20 daily injections and the animals were killed on the 24th day. Each injected group was composed of 10 animals. Each group had an average body weight of 205–218 gm. Results are shown in Fig. 1.

It is seen that increasing the number of injections has the same effect in causing a reduction in body and testes weight as giving a larger dose, probably by maintaining a high level of stilbestrol in the body. The body weight follows the same pattern as the testes weight but the differences are less pronounced.

The effect of a sustained high level of stilbestrol in the body is confirmed in the second experiment in which stilbestrol was administered orally in 0.2 cc. of Mazola Oil (corn oil) in amounts of 1, 5, 7.5, and 25 μgm. six days per week for a period of 180 days to four groups of male rats. A control group received 0.2 cc. of Mazola Oil daily. At the beginning of the experiment the average weight of the animals was 270 ± 8 gm. The resultant loss in body weight and accessory sex organ weight is shown in Fig. 2.

*Toronto Elevators Ltd.

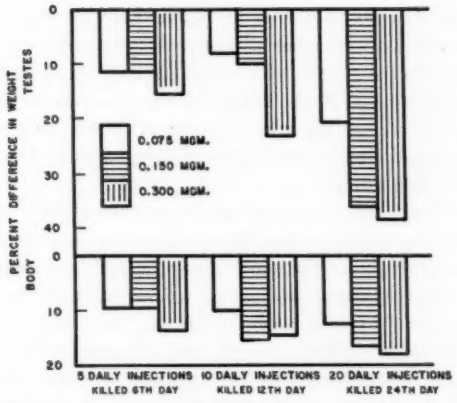


FIG. 1. The effect, on body and testes weight, of increasing the number of doses of stilbestrol.

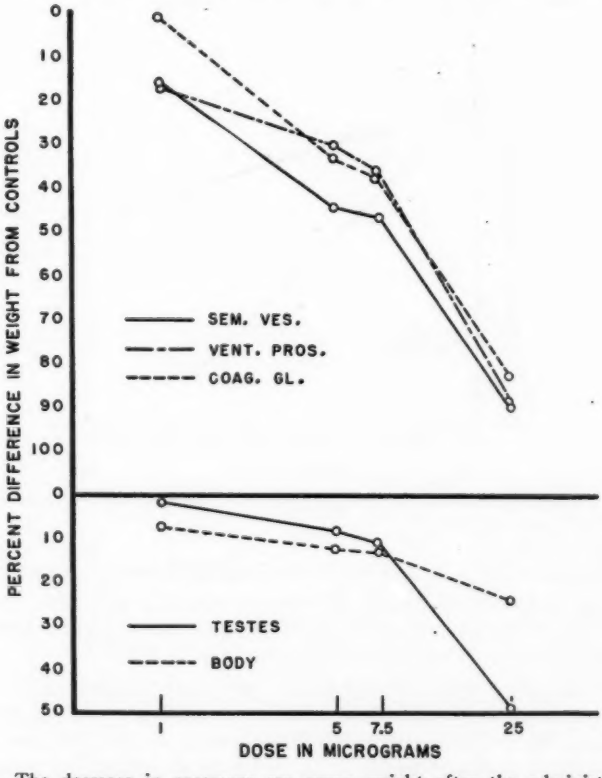


FIG. 2. The decrease in accessory sex organ weight after the administration of stilbestrol for 180 days.

It was anticipated from the work of Selye (23) on the development of a tolerance to stilbestrol that chronic administration might result in the development of a hormone resistance which would mask the effect of difference in dosage. At the end of 180 days however, the effect was the same as in the short term experiments, the greater the dosage administered the greater the loss of body weight and the weight of the testes, seminal vesicles, coagulating glands, and ventral prostate.

This particular experiment is interesting because stilbestrol given orally is one-fifth to one-seventh as effective in producing estrus in ovariectomized rats as the same amount given subcutaneously (3, 22, 19). Therefore the results obtained are the effect of extremely small amounts of the estrogen.

In the third experiment it has been shown that a relationship exists between the quantity of stilbestrol administered and the loss of weight of body and testes. In this experiment groups of five animals with an average body weight of 208 ± 2 gm. at the beginning of the experiment were injected daily for 10 days with varying doses of stilbestrol and killed on the 15th day. Each point in Fig. 3 represents five animals on one of the 13 doses given.

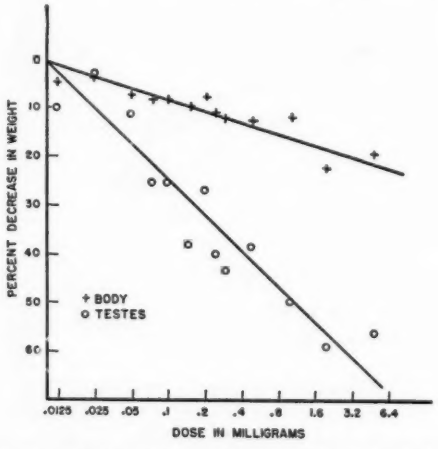


FIG. 3. Relationship between the quantity of stilbestrol administered and loss of weight of body and testes; $r = 0.904$.

It can be seen that a straight line relationship exists between the log dose and response as measured by the decrease in weight in the range 0–5 mgm.

The correlation between the loss in body weight and the loss in testes weight is highly significant. (Correlation coefficient $r = 0.904$.)

Although the decrease in weight of the body and accessory sex organs due to the administration of stilbestrol has been reported to be temporary, the time at which the weights return to normal or increase, so that they are equal to control values, was not known. In order to elucidate this point the following experiment was carried out. Groups of 10 rats, each with an average

body weight of 140 gm. were given five daily subcutaneous injections of 60 μgm. of stilbestrol. The various groups were taken off on the 1st, 20th, 25th, 46th, and 63rd day after the injections were discontinued. Groups of eight untreated rats of the same weight range were sacrificed on the same days so that a measure of the recovery of body and organ weights could be obtained.

The curves in Fig. 4 show the difference between the injected animals and the controls at these various times. It is noted that the body weight reaches that of the controls at about the twentieth day. This is in agreement with the work of Hoskins and Small (8) who found that a period of about three weeks was required to regain the weight which had been lost. The testes took somewhat longer to regain their control weight, approximately nine weeks, and

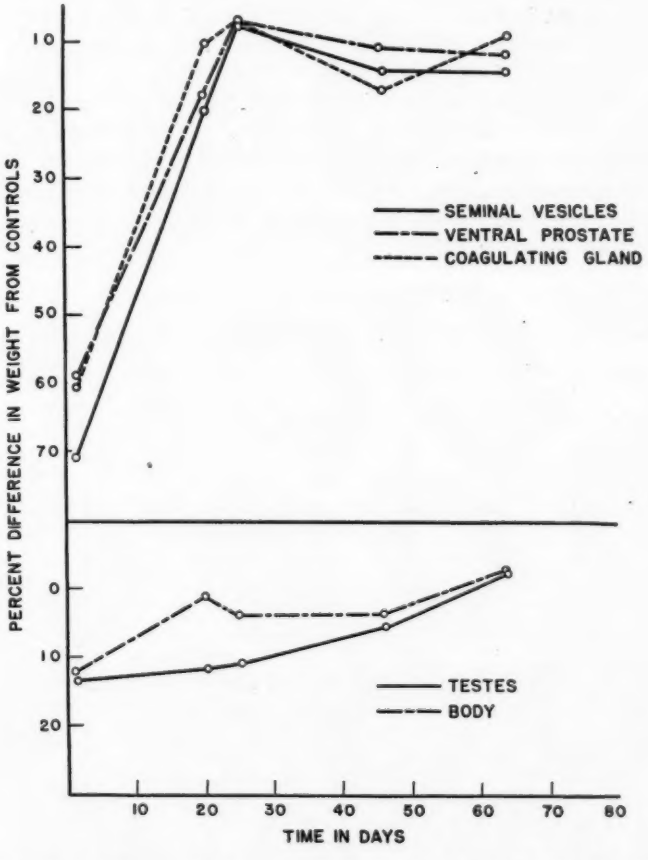


FIG. 4. Per cent recovery of body and accessory sex organ weight after stilbestrol administration was discontinued.

this is in agreement with the results of Matthews *et al.* (11) who have reported that the testes regained their functional state as measured by the siring of a litter at the end of the seventh week after stilbestrol administration was discontinued. The seminal vesicles, coagulating glands, and ventral prostate show a rapid gain to about the twenty-fifth day and then approach a plateau approximately 10% lower than control values. As this experiment was not carried beyond the 64th day it is not known at what point full recovery of these organs takes place or whether there is a permanent effect on their size and weight.

In experiment 5, adult male and female rats of the same age were paired off at random and when a sufficient number had proved their breeding ability by producing a litter, the males were segregated from the females and divided into six groups. To five of these groups stilbestrol in oil was given orally in a volume of 0.2 cc. in the following concentrations, 5, 6, 7.5, 10, and 25 μgm. The oil was given with a syringe and blunt needle, being placed on the back of the tongue to ensure that the animal would swallow it.

The doses were given to the males for five days prior to mating and then every day for the duration of the experiment. These animals were mated with the same females as before so that they would be compatible.

A record of litters was kept for 180 days at the end of which time the experiment was terminated. A control group received 0.2 cc. of corn oil daily. The results are shown in Table I.

TABLE I

EFFECT OF STILBESTROL ON THE REPRODUCTIVE CAPACITY OF THE MALE RAT

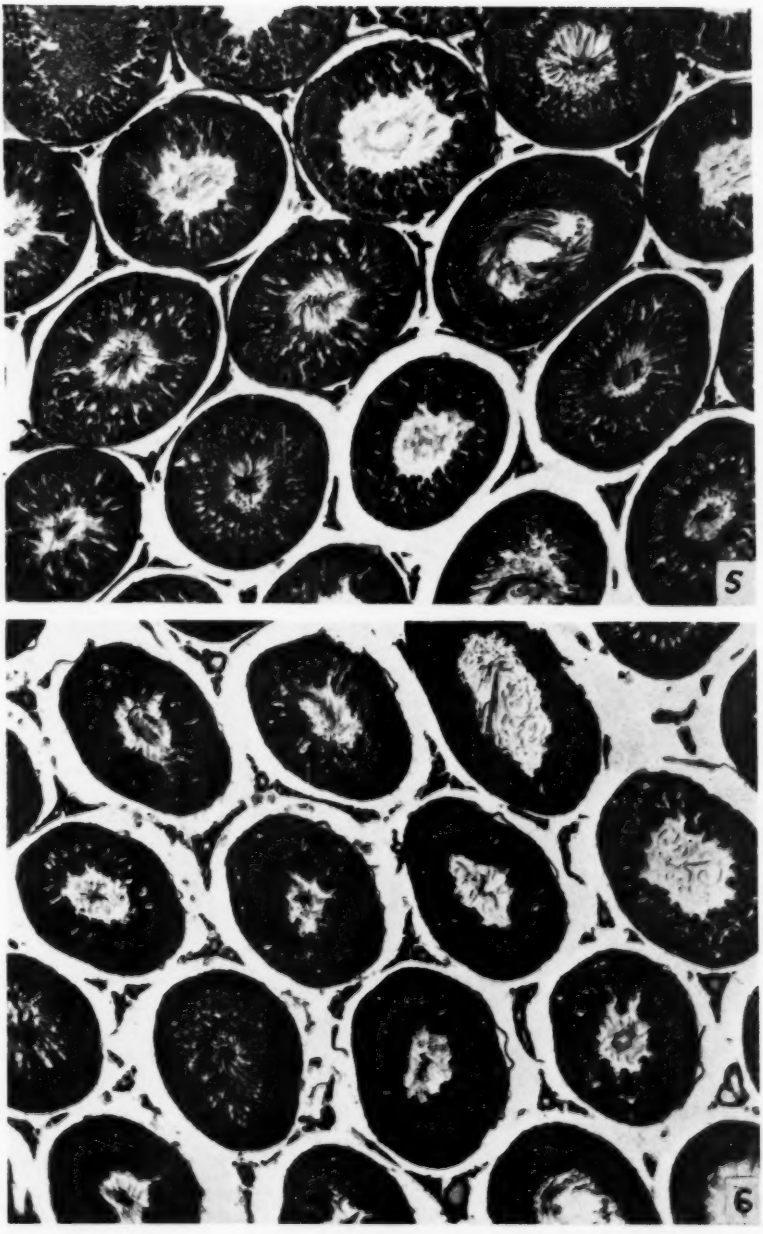
Number of pairs	Micrograms of stilbestrol	Number of litters	Average number of litters per pair	Average number in litter
8	0	23	2.88	8.96
6	5	13	2.16	7.54
6	6	14	2.33	7.43
8	7.5	1	0.13	2.0
10	10	0	0	0
10	25	0	0	0

From Table I it can be seen that a normal number of litters were produced by the control group and those receiving 5 and 6 μgm. of stilbestrol per day. However, only one litter was produced by the group on the 7.5 μgm. dose and none on the 10 and 25 μgm. doses. It appears that there is a difference,

FIG. 5. A section of normal rat testis.

FIG. 6. A section of testis of a rat receiving 6 μgm. of stilbestrol daily for 180 days.

NOTE: All sections shown in Plates I and II were cut at 4μ and stained with Weigerts iron hematoxylin and eosin stain. ×140.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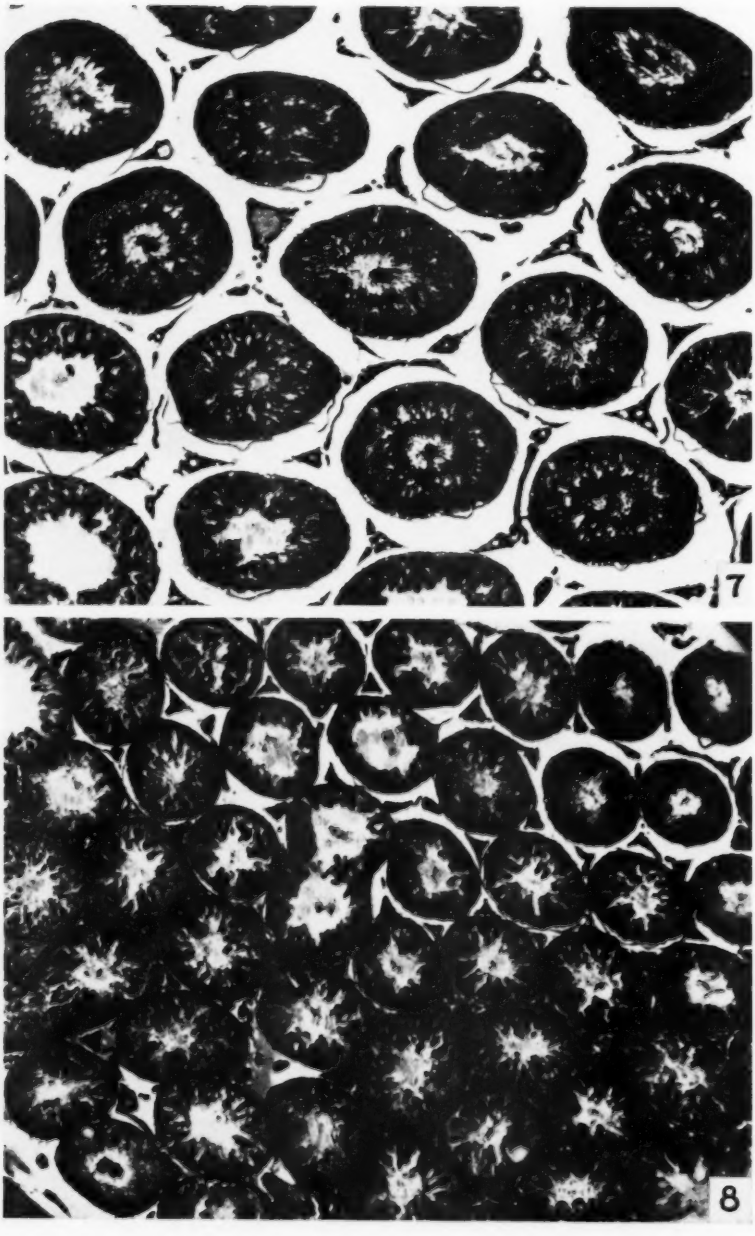
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between the control group and those receiving 5 μgm. and 6 μgm. per day, in the number of litters per pair and the average number in the litter, but these differences are not significant.

Histological examination of sections of the testes of the rats on the 5 and 6 μgm. doses show them to be normal but some of the sections from the animals receiving 7.5 μgm. per day showed degenerative changes in the tubules with cellular detritus in the lumen. However, the majority of the tubules showed normal spermatogenesis with large numbers of mature sperm.

In the group which received 25 μgm. of stilbestrol daily, spermatogenesis was incomplete in many and greatly reduced in other tubules and the tubular diameter was decreased. Many tubules showed phagocytosis of cellular detritus by free floating Sertoli cells and multinucleated giant cells.* As there appeared to be no appreciable decrease in the size of the spermatogenic cells it would seem that there was a decrease in the number of proliferating spermatogenic cells in the higher dosage level.

Histologically, sections of the prostates from the rats receiving 5, 6, and 7.5 μgm. appeared to be normal but some of those on the 25 μgm. dose showed rarefaction of the cytoplasm of the epithelial cells with pyknotic nuclei in many of these cells.

Discussion

The mechanism through which the body weight loss, due to the administration of stilbestrol, is brought about is not clear. It has been found by Richards and Kueter (20) that the inhibition of growth and loss of body weight produced by 2 mgm. of stilbestrol daily could be fully compensated for by adequate administration of growth hormone and these workers suggested that the mechanism of chronic suppression is at least partly due to inhibition of production of pituitary growth hormone in the stilbestrol treated animals. Gaarenstroom and Levie (5) on the other hand, found that growth hormone administered simultaneously with stilbestrol did not compensate for the effect of the latter on bone growth.

Richards and Kueter (20) also suggest that the actual loss of body weight which follows the arrest of growth is probably due to a decrease in the amount of food consumed by the animals, and that it appears that the administration of growth hormone stimulates the appetite to a sufficient degree to cause a marked increase in the food intake in addition to the stimulation of growth.

* For typical sections of testes on the various doses see Fig. 5, 6, 7, and 8.

FIG. 7. A section of testis of a rat receiving 7.5 μgm. of stilbestrol daily for 180 days.

FIG. 8. A section of testis of a rat receiving 25 μgm. of stilbestrol daily for 180 days.

A decrease in size of the tubules from the normal can readily be seen in the testes of the animals which received stilbestrol. In those from animals receiving 6 and 7.5 μgm. per day spermatogenesis appears normal; however, the morphological features are less distinct. In the sections from animals receiving 25 μgm. per day spermatogenesis appears to be complete only to the stage of secondary spermatocytes.

This conclusion is supported by the work of Meites (12) who carried out experiments on paired feeding and found that most of the decrease in body weight due to the administration of stilbestrol could be accounted for by a decreased food intake.

There is no direct evidence however that the loss in weight of the accessory sex organs due to stilbestrol administration is the result of a loss of appetite. Noble (17) and Greep and Jones (6) have found that this loss in organ weight in normal animals, injected with estrogen, when compared with the loss in weight produced by hypophysectomy, indicates that the gonadotrophic activity of the pituitary may be completely suppressed with a resulting atrophy of the sex organs.

In the breeding experiment the difference in the dose of stilbestrol between those which produced a normal number of litters and those which produced none is small, approximately 1.5 μgm. per day. This difference becomes very small indeed when it is considered that stilbestrol given orally is from one-fifth to one-seventh as effective, with respect to producing estrus in ovariectomized rats, as though given subcutaneously (3, 19, 22).

The reason for sterility at this dose level is not known. It may be due to a loss of libido, although it was noted that the behavior of the males towards the females in this group appeared to be the same as in the control group.

Since there is some evidence that hyaluronidase plays a part in the fertilization of the egg by the sperm (7, 18, 24), it is possible that sterility may be due to a decrease in hyaluronidase production by the testes when 7.5 μgm. of stilbestrol per day is administered. The histological picture of the testes on this dose is not different enough from the control picture to account for the loss in breeding activity by a diminution in the number of sperm, the tubules being full of apparently normal mature forms.

Although the results obtained in these experiments on the effect of the oral administration of relatively small amounts of stilbestrol on the accessory sex organs of male rats cannot be compared directly to the effect which may be produced in man as a result of the ingestion of the flesh of animals treated with stilbestrol, they do however emphasize that a degree of caution should be exercised before the practice of using stilbestrol as a conditioning supplement is instituted.

Residues containing significant amounts of stilbestrol have been recovered from the fatty tissue of poultry after the pellet implantation of this synthetic estrogen (25). In addition in feeding experiments in poultry using the dimethyl ether of stilbestrol, Bird *et al.* (1) have reported that sufficient residues of estrogen are present in the fat of such treated poultry, when fed to postmenopausal women, to cause a change in the cells of the vaginal canal as exemplified by the disappearance of the more differentiated squamous variety with varying degrees of cornification.

Acknowledgment

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FACTORS AFFECTING THE INCORPORATION OF RADIOACTIVE PHOSPHATE INTO THE PHOSPHOLIPIDS OF SLICES OF CAT BRAIN[1]

By K. P. Strickland

Abstract

Slices of cat brain respiring in a Krebs–Ringer bicarbonate medium were found to incorporate radioactive inorganic phosphate (P^{32}) into the phospholipid fraction. The addition of glucose or mannose increased the incorporation of P^{32} into the phospholipids. Fructose caused a small increase, whereas galactose was without effect. Pyruvate and lactate increased the incorporation of P^{32} into the phospholipids. Succinate, L (+)-glutamate, D (−)-glutamate, α keto-glutarate, citrate, and L-malate failed to support the incorporation.

Anaerobic conditions and homogenization of the tissue prevented the *in vitro* incorporation of P^{32} into the phospholipids of cat brain. A wide range of metabolic inhibitors (cyanide, azide, malononitrile, chloretone, nembutal, iodoacetate, and fluoride), in concentrations that inhibit the oxygen consumption of brain slices, inhibited the incorporation. The incorporation was also inhibited by 2,4-dinitrophenol in concentrations that do not decrease the oxygen consumption of brain slices.

These findings indicated that the incorporation of P^{32} into the phospholipids of slices of cat brain is a metabolic phenomenon and is dependent upon the maintenance of an adequate phosphorylating mechanism within the slice.

Introduction

The *in vitro* incorporation of radioactive inorganic phosphate into the phospholipids of brain was first reported by Chaikoff and co-workers (9, 28). They showed that slices of rat brain respiring in an oxygenated medium were able to incorporate radioactive inorganic phosphate (P^{32}) into the phospholipids, even in the absence of added substrates, although the addition of certain hexoses greatly enhanced the incorporation. A study of some of the factors affecting the incorporation of P^{32} into the phospholipids of slices of cat brain is described in this paper. The experiments indicate that a suitable oxidative phosphorylating mechanism is necessary for the incorporation of P^{32} into the phospholipids of brain slices.

A preliminary report of these experiments has already appeared (5).

Methods

Preparation and Incubation of Tissues

The cerebral hemispheres were removed from a cat as soon as possible after death by decapitation. Slices of brain tissue were then prepared free-hand using a safety razor blade. Each slice was rapidly weighed on a torsion balance and transferred to a 15 ml. Warburg vessel containing a 5 ml. Krebs–Ringer bicarbonate incubating medium, to which had been added sufficient glucose to give a final concentration of 0.2%. About 500 mgm. of slices were placed in each flask. In some experiments, glucose was omitted from the incubating

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medium, or replaced by other substrates. Solutions of substrates and inhibitors were freshly prepared in Krebs–Ringer bicarbonate. When necessary, the solution was titrated to the pH (7.4) of the buffer with hydrochloric acid or sodium hydroxide. To each flask was then added 24 μc. of radioactive phosphate* (P^{32}). The flasks were aerated for 10 min. with 95% O_2 – 5% CO_2. The vessels were then incubated at 37.2° C., with shaking, for four hours. For anaerobic experiments, the flasks were aerated with 95% N_2 – 5% CO_2, which had been passed over hot copper fillings to remove the last traces of oxygen. At the end of the incubation period, the contents of each flask were homogenized in 10% trichloroacetic acid at 0° C. with a Potter–Elvehjem homogenizer. For the unincubated samples (0-hr. incubation times), the radioactive phosphate was added to the vessels containing the brain slices. The contents of the flasks were then well mixed and the brain homogenized in the presence of the P^{32}, as described for the incubated samples.

Determination of the Specific Activity of the Phospholipids

The acid-soluble phosphorus compounds were removed by 10 extractions with 5 ml. portions of 10% trichloroacetic acid at 2–4° C. Seven of these extractions were carried out with 10% trichloroacetic acid containing 0.04 *M* potassium dihydrogen phosphate. The inactive phosphate was added as a carrier to aid in the removal of the last traces of the very active inorganic phosphate that surrounded the brain slices during incubation.

The methods for the extraction, purification, and measurement of the specific activity of the phospholipids have been described in a previous paper (32). The results have been corrected for decay and calculated on the basis of a standard count of 8.1×10^5 impulses/min./ml. incubating medium. Each value is the average of duplicate samples.

Results

Glucose as Substrate

Table I shows the specific activities obtained for the phospholipids of brain slices incubated in the presence of inorganic P^{32}. The low figures for the 0-hr. slices indicate that very little of the activity of the 4-hr. slices could be due to contamination of the lipid with inorganic P^{32}, since the 0-hr. slices were suspended in the medium containing the same amount of P^{32} as the incubated samples and they were then homogenized in the presence of the P^{32}. Glucose, which is known to stimulate oxygen uptake of brain tissue *in vitro* (17, 27, 3), considerably increased the incorporation of P^{32} into the phospholipids. The specific activity of the phospholipids was found to increase as the glucose concentration was increased from 0-100 mgm./ml., and it then changed little as the glucose concentration further increased to 800 mgm./100 ml. The stimulatory effect of glucose on the incorporation of P^{32} into the phospholipids of slices of cat brain is similar to that already demonstrated by Schachner, Fries, and Chaikoff (28) for slices of rat brain.

* *Radioactive phosphate in the form of $H_3P^{32}O_4$ was received from the Eldorado Mining and Refining (1944) Limited.*

TABLE I

SPECIFIC ACTIVITY OF THE PHOSPHOLIPIDS OF SLICES OF CAT BRAIN INCUBATED IN KREBS–RINGER BICARBONATE BUFFER CONTAINING GLUCOSE AND P^{32}

(Counts per min. per μgm. P)

	0 hr.	4 hr.	4 hr. Glucose omitted
Expt. 1	0.3	19.1	5.7
Expt. 2	0.2	11.0	4.1
Expt. 3	0.0	19.5	6.1
Expt. 4	0.3	14.3	4.4
Expt. 5	0.2	16.2	5.6

In Fig. 1 a specific activity – time curve for the phospholipids is shown along with the curve for pentosenucleic acid that was reported in a previous paper (4). The specific activity of the phospholipids increased more rapidly than the specific activity of the pentosenucleic acid for the first hour of incubation. The specific activity curve of the phospholipids then ceased to be linear, whereas the specific activity of the pentosenucleic acid increased linearly as the incubation time was increased up to four hours.

Table II shows that anaerobic conditions almost completely abolished the incorporation of P^{32} into the phospholipids. The incorporation was also much

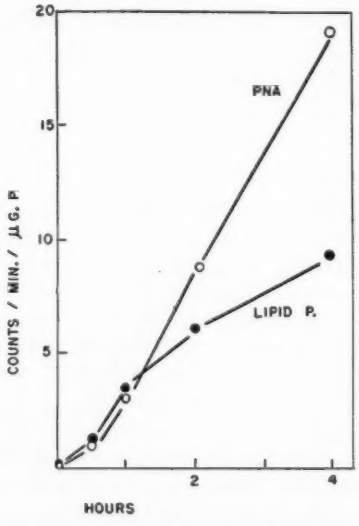

FIG. 1. Specific activity of the phospholipids (lipid P) and pentosenucleic acid (PNA) of slices of cat brain incubated at 37.2° C. in Krebs–Ringer bicarbonate containing P^{32}. Glucose, 0.011 *M*. Abscissa, hours (incubation time). Ordinate, counts per min. per μgm. P (specific activity).

TABLE II

EFFECT OF ANAEROBIC CONDITIONS ON THE SPECIFIC ACTIVITY OF THE PHOSPHOLIPIDS OF SLICES OF CAT BRAIN INCUBATED AS DESCRIBED IN TABLE I

(Counts per min. per μgm. P)

	Expt. 1	Expt. 2
95% O_2 – 5% CO_2	19.1	20.4
95% N_2 – 5% CO_2	1.6	0.6
Percentage inhibition	92	97

TABLE III

SPECIFIC ACTIVITY OF THE PHOSPHOLIPIDS OF HOMOGENATES* OF CAT BRAIN INCUBATED IN KREBS–RINGER BICARBONATE BUFFER CONTAINING GLUCOSE AND P^{32}

(Counts per min. per μgm. P)

—	Expt. 1	Expt. 2	Expt. 3	Expt. 4
0 hr.	0.9	0	0.2	0.3
4 hr.	2.1	3.4	2.9	2.8

* *Each flask contained (in final concentration): glucose, 0.011 M; diphosphopyridine-nucleotide, 0.004 M; adenosinetriphosphate, 0.004 M; sodium fluoride, 0.025 M. The brain was homogenized in Ringer containing 0.04 M nicotinamide. Incubation time, 4 hr.*

less when the cell structure of the brain was disrupted by homogenization (Table III). This decreased incorporation occurred despite the addition of several 'reinforcing' factors, such as diphosphopyridinenucleotide and adenosinetriphosphate, and the addition of sodium fluoride, which inhibits the breakdown of adenosinetriphosphate. Each of the additions was found to increase the incorporation, but the activity of the full system was much less than that given by the slices.

Other Substrates

The effect of a number of monosaccharides is shown in Table IV. Mannose gave an incorporation as great as glucose, but fructose was less effective. Galactose did not increase the specific activity beyond that obtained without the addition of substrate. This result is somewhat different from the known effects of these substrates on the oxygen consumption of brain *in vitro* (17, 27, 3). Both mannose and fructose are as active as glucose in increasing the oxygen uptake, whereas galactose is reported to give a smaller increase (27,3) or no increase (17).

TABLE IV

EFFECT OF MONOSACCHARIDES ON THE SPECIFIC ACTIVITY OF THE PHOSPHOLIPIDS OF SLICES OF CAT BRAIN INCUBATED AS DESCRIBED IN TABLE I

(Counts per min. per μgm. P)

	Without substrate	With substrate	Percentage increase
Glucose 0.011 *M*	4.2	11.8	180
	6.2	21.0	238
	3.6	11.7	227
	4.4	10.6	142
	3.6	9.0	151
Mannose 0.01 *M*	4.2	12.2	190
	6.2	22.0	254
	3.6	10.6	196
	3.6	8.2	128
Fructose 0.01 *M*	4.2	6.8	61
	6.2	11.1	77
	3.6	5.1	43
	3.6	5.5	52
Galactose 0.01 *M*	4.2	3.8	−10
	6.2	6.7	8
	3.6	3.6	1
	4.4	4.6	5

TABLE V

EFFECT OF PYRUVATE AND LACTATE ON THE SPECIFIC ACTIVITY OF THE PHOSPHOLIPIDS OF SLICES OF CAT BRAIN INCUBATED AS DESCRIBED IN TABLE I

(Counts per min. per μgm. P)

	Without substrate	With substrate	Percentage increase
Pyruvate 0.01 *M*	4.1	5.5	36
	6.1	8.3	35
	4.4	5.3	20
Lactate 0.01 *M*	4.4	6.4	46
	3.6	3.7	2
	4.4	5.7	30
	3.6	4.4	21

Table V indicates that the addition of either pyruvate or lactate increased the incorporation of P^{32} into the phospholipids, but only to a limited extent compared with glucose. Each of these substrates are known to increase the oxygen consumption of brain *in vitro* (17, 27, 3).

TABLE VI

EFFECT OF OTHER SUBSTRATES ON THE SPECIFIC ACTIVITY OF THE PHOSPHOLIPIDS OF SLICES OF CAT BRAIN INCUBATED AS DESCRIBED IN TABLE I

(Counts per min. per μgm. P)

	Without substrate	With substrate	Percentage increase
Succinate 0.05 *M*	4.1	3.7	− 9
	6.1	5.5	−10
L (+)-Glutamate 0.01 *M*	4.1	4.0	− 2
	6.1	5.9	− 3
	4.4	4.4	− 1
	4.4	4.9	11
D (−)-Glutamate 0.01 *M*	4.4	4.5	2
α-Ketoglutarate 0.01 *M*	6.0	7.2	20
Citrate 0.01 *M*	6.0	6.6	9
Malate 0.01 *M*	6.0	6.3	5

Table VI shows the effect of several other substrates. Neither succinate nor L (+)-glutamate increased the incorporation, although each of these substrates supports oxygen consumption (27, 16, 3). D(−)-glutamate, which inhibits oxygen uptake (36), did not increase the incorporation. The addition of the tricarboxylic acid cycle intermediates, citrate, α-ketoglutarate, or malate was also without effect on the incorporation of P^{32} into the phospholipids.

Inhibitors

In Table VII is shown the effect of a number of metabolic inhibitors on the incorporation of P^{32} into the phospholipids of brain slices. With the exception of the lower concentrations of 2,4-dinitrophenol, all the inhibitors were used in concentrations that are known to decrease the oxygen consumption of the slice. For all the inhibitors tested there was a marked decrease in the incorporation, with the exception of 10^{-5} *M* 2,4-dinitrophenol and 10^{-2} *M* malonate.

Discussion

The findings on the incorporation of P^{32} into the phospholipids of slices of cat brain are in general agreement with the findings of Chaikoff and associates for rat brain. The hexoses, glucose, and mannose, were found to cause large increases in the incorporation. Schachner *et al.* (28) reported similar increases with these hexoses. However, contrary to the findings reported here, these workers found that fructose and galactose were equally effective.

Several reasons may account for the small effects observed with fructose and galactose. Neither a specific galactokinase nor a specific fructokinase

TABLE VII

EFFECT OF INHIBITORS ON THE SPECIFIC ACTIVITY OF THE PHOSPHOLIPIDS OF SLICES OF CAT BRAIN INCUBATED AS DESCRIBED IN TABLE I

(Counts per min. per μgm. P)

	Without inhibitor	With inhibitor	Percentage inhibition
Cyanide 3×10^{-3} *M*	7.7	0.5	93
	19.1	1.2	93
	23.5	1.6	94
Azide 10^{-3} *M*	13.7	1.1	92
	20.4	0.6	97
Malononitrile 10^{-3} *M*	18.9	2.2	88
	21.3	3.6	83
Chloretone 4×10^{-3} *M*	13.7	6.4	53
	20.4	5.5	73
Nembutal 10^{-3} *M*	13.7	7.9	42
	9.9	4.3	57
Iodoacetate 10^{-3} *M*	12.6	1.6	87
	9.9	1.4	86
Fluoride 10^{-2} *M*	20.4	3.1	85
2,4-Dinitrophenol 10^{-3} *M*	12.6	0.7	95
	18.9	1.0	95
10^{-4} *M*	12.6	1.6	87
	18.9	1.4	93
10^{-5} *M*	12.6	11.1	12
	18.9	15.2	20
Malonate 10^{-2} *M*	13.7	16.0	−17
	9.9	9.7	2

has been demonstrated in brain (29). Brain hexokinase does not utilize galactose, but it can utilize fructose (29). However, if equimolar concentrations of fructose and either glucose or mannose are present, there is almost a 100% inhibition of fructose utilization (30). The inhibition is still 85% when fructose is present in four times the concentration of either glucose or mannose. It is possible that enough residual substrate is present in the brain slices to cause a partial inhibition of the utilization of fructose. These effects on hexose utilization by brain hexokinase preparations may explain the differences observed in the incorporation of P^{32} into the phospholipids of brain slices. They do not explain, however, why fructose increases the oxygen uptake of brain slices. It may be that fructose can be oxidized by some other pathway that cannot support P^{32} incorporation.

Although both glutamate and succinate are known to increase the oxygen uptake of brain slices, they failed to increase the incorporation of P^{32} into the phospholipids. As pointed out by Weil-Malherbe (37, 38), it is unlikely that

L (+)-glutamate is a major energy-yielding substrate for brain tissue. In support of this McIlwain (20, 21) has shown that glutamic acid, unlike glucose, was unable to maintain the level of phosphocreatine in slices of rat brain and guinea pig brain respiring aerobically. It is probable that succinate also is not a major energy-yielding substrate, for it failed to support the aerobic formation of acetylcholine by brain slices (25), it failed to support the active transport of glutamate against a concentration gradient (31), and it failed to maintain the level of phosphocreatine in brain slices (15).

Anaerobic conditions and homogenization of the tissue greatly decreased the incorporation in the experiments reported here and in those reported by Schachner *et al.* (28). The marked inhibition noted with the metabolic inhibitors cyanide and azide is similar to that reported by Taurog, Chaikoff, and Perlman (33) for the incorporation of P^{32} into the phospholipids of slices of kidney and liver of the rat.

Malonate inhibits the oxidation of succinate in brain slices by competing with the succinate for the enzyme, succinic dehydrogenase (26). The oxidation of glucose is also inhibited by malonate in slices of rat brain (11), probably by the competitive inhibition of succinic dehydrogenase, which is necessary for complete oxidation of glucose through the tricarboxylic acid cycle. The finding that malonate did not inhibit the incorporation of P^{32} into the phospholipids indicates that the oxidations beyond succinate are not required for the incorporation. This is consistent with the finding that the addition of succinate had no effect on the incorporation.

With the exception of 2,4-dinitrophenol, most of the inhibitors (in the concentrations used) probably affected the incorporation of P^{32} into the phospholipids as a result of their inhibition of respiration. Since 2,4-dinitrophenol is representative of a group of metabolic inhibitors whose mode of action may be different, this substance was studied over a wide range of concentrations. Loomis and Lipmann (18) reported that low concentrations ($10^{-4} - 10^{-5}$ *M*) of dinitrophenol inhibited the uptake of inorganic phosphate, but not of oxygen, by washed homogenate preparations from kidney. Although there is still considerable doubt as to the mechanism whereby dinitrophenol exerts its effect (34, 13), subsequent work, in a number of laboratories with a wide variety of tissue preparations, including brain homogenates (1), has shown that dinitrophenol is one of a series of substances that may prevent the formation of energy-rich phosphate compounds without decreasing oxygen consumption. The incorporation of P^{32} into the phospholipids of cat brain slices was greatly inhibited by dinitrophenol in a concentration of 10^{-4} *M* and the incorporation was slightly inhibited by a concentration as low as 10^{-5} *M*. Dinitrophenol, in this concentration range, stimulates oxygen consumption of brain slices (24, 35, 23). The degree of the inhibition of the incorporation of P^{32} into the phospholipids was of the same order as that reported by Frantz, Zamecnik, Reese, and Stephenson (6) for the incorporation of C^{14}-alanine into the proteins of liver slices of the rat.

The evidence with dinitrophenol indicates that, when the formation of energy-rich phosphate bonds is inhibited, the incorporation of P^{32} into the phospholipids is inhibited. Kennedy (14) has reported the necessity of oxidative phosphorylation (formation of energy-rich phosphate compounds) for the incorporation of P^{32} into the phospholipids of rat liver mitochondria. It is possible that the P^{32} must be incorporated into the labile energy-rich phosphate compounds as a prerequisite to incorporation into the phospholipids. A number of workers (7,8,10,12) have shown that under suitable *in vitro* conditions P^{32} is readily incorporated into the labile phosphate compounds of other tissues.

Factors that affect the incorporation of P^{32} into brain slices also affect a number of other synthetic reactions in a similar way. The formation of acetylcholine by brain slices under aerobic conditions (25) is also decreased by anaerobiosis, homogenizing the tissue or by the addition of metabolic inhibitors to the respiring slices. The addition of a substrate such as glucose, mannose, lactate, or pyruvate increases the acetylcholine formation (19) but fructose, galactose, or succinate are without effect. There is thus a similarity in the conditions that will support either the incorporation of P^{32}, or the formation of acetylcholine, in brain slices.

Conditions that adversely affect the incorporation of P^{32} into the phospholipids have been found by McIlwain and his associates to be responsible for the failure of guinea pig brain slices to maintain the level of phosphocreatine. The absence of oxygen or glucose (22, 21), replacing the glucose by L (+)-glutamate (20, 21) or succinate (15), or the presence of a wide range of metabolic inhibitors are all factors that are responsible for the failure of both guinea pig brain slices to maintain the concentration of phosphocreatine and cat brain slices to incorporate P^{32} into the phospholipids. Dinitrophenol, which may affect brain metabolism differently (23, 21), also inhibits phosphocreatine formation and the incorporation of P^{32}.

Recently, DeLuca, Rossiter, and Strickland (2) showed that P^{32} was similarly incorporated into the pentosenucleic acid of brain slices. The factors that affect the incorporation were found by Findlay, Rossiter, and Strickland (4) to be very similar to those affecting the incorporation of P^{32} into the phospholipids suggesting that similar mechanisms are involved. These experiments with pentosenucleic acid and the experiments reported here with phospholipids are consistent with the view that the incorporation of P^{32} is dependent upon: (1) aerobic conditions within the cell, since anaerobiosis and metabolic inhibitors (in concentrations that inhibit respiration) prevented the incorporation, (2) energy-producing substrates, since glucose, mannose, lactate, and pyruvate increased the incorporation, and (3) the formation of energy-rich phosphate bonds, since in circumstances where the formation of energy-rich bonds is inhibited, the incorporation of P^{32} into both the phospholipids and the pentosenucleic acid is also inhibited.

Acknowledgments

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THE PROTEOLYTIC ENZYMES OF MICROORGANISMS

VII. A STUDY OF SOME OF THE PROPERTIES OF TWO PROTEASES ISOLATED FROM *MORTIERELLA RENISPORA* DIXON-STEWART[1]

By L. R. Wetter

Abstract

Two proteases isolated from the culture medium of *Mortierella renispora* Dixon-Stewart (PRL 26) had different isoelectric points but similar pH optimums. The components were inhibited by equal amounts of ovomucoid. Heat studies showed that the constituent moving towards the cathode (cathodic component) was more stable at 58° C. than the anodic component. The enzyme located in the cathodic component hydrolzyed chloroacetyl-L-tyrosine. The same fraction also hydrolyzed N-carbobenzoxy-α-L-glutamyl-DL-alanine and to a much lesser extent N-carbobenzoxy-α-L-glutamyl-L-glutamic acid. No enzymatic activity could be detected against glycylglycine, diglycylglycine, glycyl-L-proline, seryl-serine, or α-benzoyl-L-argininamide.

Introduction

A number of years ago Johnson (5) showed that the mycelial extracts of *Aspergillus parasiticus* contained at least four protein-hydrolyzing compounds, three of which hydrolyzed different synthetic substrates. Since then Berger *et al.* (2) have tested the mycelium of a considerable number of molds for proteolytic activity. Recently McConnell and co-workers (8) have shown that the culture media from fungi grown in submerged culture possess enzymes that will hydrolyze synthetic dipeptides and their derivatives. They also observed that differences existed between different organisms.

Recently Wetter (13) has shown that a protease-rich concentrate obtained from the culture medium of *Mortierella renispora* Dixon-Stewart (PRL 26) contained two proteases both of which hydrolyzed urea-denatured hemoglobin. The present communication is a continuation of this work and shows that the two proteases differ from each other when comparisons are made with synthetic substrates. Other differences are also reported.

Experimental

Methods

The proteolytic activity was determined by using urea-denatured hemoglobin as the substrate and following the rate of hydrolysis as previously described (12, 13). The hydrolysis rate of the synthetic substrates was determined by following the release of free amino acids (7). A modification of the Conway micro-diffusion technique for ammonia (11) was used to study the hydrolysis of α-benzoyl-L-argininamide. The synthetic substrates were dissolved in 0.25 *M* phosphate buffer (pH 7.2) so that the substrate concentration in the digestion mixture was 0.05 *M*. Alkali (sodium hydroxide) was added when necessary to ensure complete solution. The

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enzyme was dissolved in 0.25 *M* phosphate (pH 7.2). The high buffer concentration was employed so that no pH changes would occur during hydrolysis. All enzymatic reactions were carried out in a water-bath maintained at 25° C. ± 0.5°.

The inhibitor studies were carried out by adding known quantities of ovomucoid to the enzyme solution. The latter was made up in 0.1 *M* phosphate buffer (pH 7.6) in all cases. The mixture of enzyme and inhibitor was allowed to stand at room temperature for 10 min. (6), and then added to the hemoglobin substrate for proteolytic activity measurements in the usual manner.

The pH optimum curves were obtained as described in a previous publication (12). The heat inactivation studies were carried out by heating the enzyme solution for exactly five minutes at carefully controlled temperatures. After cooling in an ice-water bath for 15 min., the enzyme activity was determined.

Materials

The enzymes from a partially purified culture medium of PRL 26 (12) were isolated by zone electrophoresis as described elsewhere (13). The component designated as the "anodic component" travelled towards the anode while the "cathodic component" moved towards the cathode. These fractions were isolated on starch slabs extracted with saline, then freeze-dried and stored at 0° C. until ready for further use.

The egg-white inhibitor, ovomucoid, was prepared from fresh hen's eggs by the method of Lineweaver and Murray (6). The α-benzoyl-L-argininamide and the chloroacetyl-L-tyrosine were purchased from Delta Chemical Works, New York 23, N.Y. Glycyl-L-proline, glycylglycine, and diglycylglycine were obtained from Mann Research Laboratories, New York 6, N.Y. The N-carbobenzoxy derivatives were supplied by Mr. J. A. Trew (8).

Results and Discussion

pH Optimum

The effect of hydrogen ions on the activity of the enzyme is shown in Fig. 1. The anodic component has an optimum which is similar to that obtained for the original material. The cathodic component appears to be equally broad although its optimum has shifted approximately 1.5 pH units towards the alkaline side.

It is interesting to note that the two electrophoretic components have the same wide pH optimum. Adams and Smith (1) showed that a broad pH optimum in the case of proteolytic activity in pituitary extracts indicates the presence of at least two enzymes. This does not appear to be the case in the present study as both of the electrophoretically isolated components have wide pH optimums. Another point of interest is that the pH-activity curves for the above components are in the range found for most proteolytic enzymes excepting pepsin and the cathepsins.

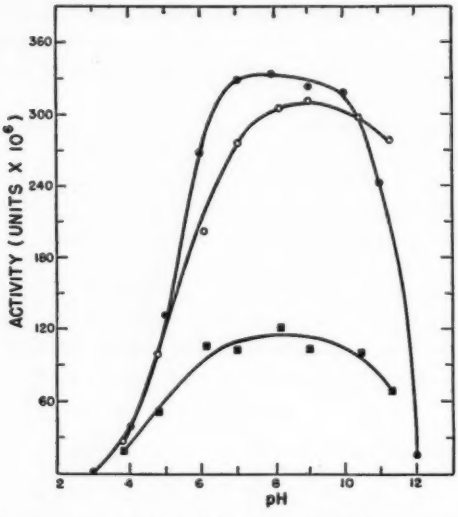

FIG. 1. pH optimum curves of (1) original concentrate ●——●, (2) anodic component ■——■, and (3) cathodic component ○——○.

Inhibition by Ovomucoid

The existence of a proteolytic inhibitor in egg white has been known for some time. Recently it was shown that this inhibitor is ovomucoid (6) and that it greatly depressed the action of trypsin. Though other inhibitors exist, very little is known about the comparative effects of any single one on different proteolytic enzymes. We have observed that egg white suppresses the proteolytic activity of the culture medium of molds. If the action of an inhibitor varied for different proteases, one might use it to distinguish between different proteolytic fractions. Results presented in Table I show that 50% inhibition is obtained when between 1 and 2 mgm. of ovomucoid is added to the enzyme mixture but no differences are evident between the two fractions. Since considerable differences do exist between the two components as will be shown later, one can conclude that in this case ovomucoid is not a specific inhibitor.

TABLE I

THE PERCENTAGE INHIBITION OF THE CATHODIC AND ANODIC FRACTIONS BY OVOMUCOID

Mgm. ovomuciod per ml.	% of activity left	
	Cathodic component	Anodic component
0.00	100	100
0.48	73	75
1.04	55	59
2.0	45	41
4.0	43	41

Effect of Heat and pH

It was thought that perhaps the anodic and cathodic fractions could be further distinguished by their behavior towards heat. The samples were heated as indicated (see Methods) and the results in Fig. 2 show that the rate of destruction is less for the cathodic fraction than for the anodic one. In the former, over the ten degree range (55° C. to 65° C.) destruction of activity takes place at about 6% per degree rise in temperature. However, the anodic fraction suffers about 12% loss per degree rise over a narrower and lower range of temperature (52° C. to 58° C.). The greatest difference appears to

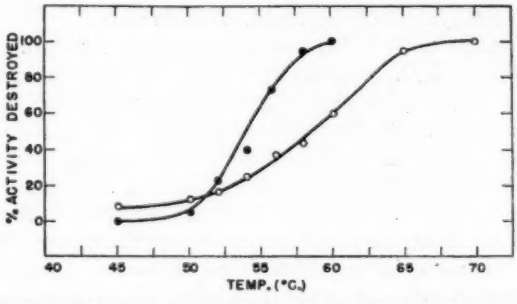

FIG. 2. The effect of heat on the stability of the two fractions isolated from PRL 26. ○———○ the cathodic component and ●———● the anodic component.

exist in a region around 58° C. where only 45% of the activity is destroyed in the cathodic fraction but very nearly 100% in the other. At this point (58° C.) and under these conditions (phosphate buffer pH 6.0 ionic strength 0.1) one could conceivably obtain the enzyme(s) in the cathodic component virtually free from the active anodic one.

Although pH often plays a profound role in the heat stability of an enzyme, the differences observed for the fractions studied were not great. Table II shows results obtained with various hydrogen ion concentrations at 55° C.

TABLE II

THE EFFECT OF pH ON THE STABILITY OF ELECTROPHORETICALLY SEPARATED COMPONENTS OF PRL 26

pH	Percentage activity left after five minutes heating at 55° C.	
	Cathodic component	Anodic component
5.5	73	48
(6.0)	(71)	(45)
6.4	69	44
7.0	79	39
7.6	67	30
8.1	58	10
8.8	32	0
9.4	15	0

Again the cathodic component is more stable than the anodic fraction, especially at more alkaline pH's. One might effect a fair separation of the two components at a pH of approximately 8.1.

Hydrolysis of Synthetic Substrates

The choice of synthetic substrates for this study is difficult, as nothing is known of the specificity of the proteolytic enzymes involved in the present investigation. The work of Johnson and co-workers (2, 5) showed that the mycelial extract of fungi hydrolyze synthetic substrates. Recently Muggleton and Webb (9) reported that the culture filtrate of *Actinomyces* sp. very rapidly attacked the amide group of α-benzoyl-L-argininamide.

The first substrate to be tested was the N-substituted argininamide. Since this compound is readily hydrolyzed by trypsin, the latter was used for comparison purposes. The trypsin used was a crystalline product* and the mold enzyme was a concentrate (See Experimental). The concentration of the enzyme solutions was such that their rate of hydrolysis of hemoglobin was equal. Table III shows that the mold enzymes do not cause any significant hydrolysis of the synthetic substrate. These results are quite different from those obtained by Muggleton and Webb who demonstrated that the culture filtrate of an *Actinomyces* sp. hydrolyzed α-benzoyl-L-argininamide more rapidly than trypsin did. It can be concluded that the proteolytic enzymes isolated from PRL 26 differ from those of *Actinomyces* sp. and that they are not trypsin-like in their behavior.

TABLE III

HYDROLYSIS OF α-BENZOYL-L-ARGININAMIDE BY TRYPSIN AND PRL 26

Time (min.)	% Hydrolysis	
	Trypsin	PRL 26
98	40	0
276	63	0
455	71	1
1405	76	2

A substrate, chloroacetyl-L-tyrosine, which is specific for carboxypeptidase, was used for further testing. Berger (2) had shown that this substrate was hydrolyzed by a large number of mycelial extracts of molds. Recently, McConnell *et al.* (8) have demonstrated that still another synthetic compound was hydrolyzed by the culture medium obtained from various fungal fermentations. This was N-cbzo**-α-L-glutamyl-DL-alanine which can be classed as a carboxypeptidase substrate because it contains a free carboxyl group and a blocked amino group. It was found that both of these substrates were hydrolyzed by protease-rich concentrates of PRL 26.

* *Armour Laboratories, Chicago, Ill. (Lot 9041X)*

** *"cbzo" is the abbreviation used for "carbobenzoxy" throughout this report.*

Since there is considerable evidence in the literature (3, 4) that the hydrolysis of substrates by proteinases follow first order kinetics, it was decided to investigate the kinetics of the enzymes of PRL 26. If there is no appreciable inactivation of the enzyme during the course of the reaction and if the products do not inhibit the reaction, first order kinetics will be found to exist (3). It was found that the hydrolysis rate for chloroacetyl-L-tyrosine and N-cbzo-α-L-glutamyl-DL-alanine (CGA) followed a first order reaction (Table IV). The relationship $K = 1/t \log \frac{a}{a - x}$ was employed, where t = time (min.), a = initial substrate concentration and x = quantity reacting in t min. The percentage hydrolysis of chloroacetyl-L-tyrosine is much higher than for CGA which probably is due to the presence of the D-isomer of alanine in the latter which is hydrolyzed at a much lower rate (8). It will be noted that the first order reaction does not hold for the entire five hour period where chloroacetyl-L-tyrosine is concerned. This suggests that the products of hydrolysis are inhibiting the reaction. In the case of CGA, K is constant suggesting that there is no inhibition by the digestion products and no inactivation of the enzyme.

TABLE IV

KINETICS OF HYDROLYSIS OF CHLOROACETYL-L-TYROSINE AND N-CBZO-α-L-GLUTAMYL-DL-ALANINE BY PRL 26

Time (min.)	Chloroacetyl-L-tyrosine		N-cbzo-α-L-glutamyl-DL-alanine	
	Hydrolysis (%)	K	Hydrolysis (%)	K
60	9.9	.00075	—	—
120	18.8	.00076	6.7	.00025
180	23.3	.00065	7.9	.00020
240	29.9	.00065	10.5	.00020
300	32.1	.00056	14.5	.00023

Since the above substrates were hydrolyzed, the anodic and cathodic components were tested to ascertain whether any differences existed. The concentrations of these components were adjusted so that each gave equal hydrolysis of hemoglobin. The cathodic component hydrolyzed both substrates approximately six times more rapidly than did the anodic component. (Table V). The percentage hydrolysis of N-cbzo-α-L-glutamyl-DL-alanine is about half of that obtained for chloroacetyl-L-tyrosine.

This raises several interesting questions. First, does the enzyme that digests denatured hemoglobin also hydrolyze chloroacetyl-L-tyrosine and/or N-cbzo-α-L-glutamyl-DL-alanine? At the present time it is not possible to determine whether we are dealing with more than one enzyme. It is conceivable that the same enzyme could be involved in the hydrolysis of hemoglobin and chloroacetyl-L-tyrosine. Second, is the same enzyme involved in

TABLE V

HYDROLYSIS OF CHLOROACETYL-L-TYROSINE (CAT) AND N-CBZO-α-L-GLUTAMYL-DL-ALANINE (CGA) BY ELECTROPHORETICALLY SEPARATED FRACTIONS

Test material	Substrate	Time	
		340	1330
Original	CAT	4.5*	15.4
	CGA	3.7	9.1
Cathodic	CAT	16.0	35.2
	CGA	8.3	16.5
Anodic	CAT	1.5	6.5
	CGA	1.3	2.1

** 156 min.*

the hydrolysis of the synthetic substrates? Both have the following in common, a blocked amino group and a free carboxyl group. This fact presents strong evidence that the cathodic component contains a carboxypeptidase. Reports in the literature (10) on the hydrolysis of synthetic substrates seriously challenge the idea of absolute specificity for many proteolytic enzymes. If this is the case, it is quite possible that only one enzyme is involved in the cathodic fraction. However, we must wait for further studies before this question can be completely answered.

The availability of several N-cbzo-α-L-glutamyl substituted amino acids made a preliminary study of specificity possible. Though the number was small, some interesting results were obtained. It can be seen from Table VI that specificity is important in these substituted peptides. A substitution of L-glutamic acid for DL-alanine resulted in a decided decrease in the hydrolysis rate. When glycine replaced the alanine no measurable hydrolysis occurred. It appears that the free amino group is important. The greatest hydrolysis arises from one which contains the methyl group as found in alanine. If

TABLE VI

COMPARISON OF THE HYDROLYSIS OF VARIOUS SUBSTRATES RELATED TO N-CBZO-α-L-GLUTAMYL-DL-ALANINE BY PRL 26

Substrate	Per cent hydrolysis at	
	330 min.	1320 min.
N-cbzo-α-L-glutamyl-DL-alanine	3.7 (7.4)*	9.1 (18.2)
N-cbzo-α-L-glutamyl-L-glutamic acid	1.0	5.0
N-cbzo-α-L-glutamyl glycine	0	0

** Value in bracket assumes that only the L-isomer is hydrolyzed.*

this group is absent as in the case of glycine there is no activity. However, if this group is lengthened as in the case of glutamic acid some activity exists, thus indicating that the length of this side chain is also important.

During the course of this investigation evidence was obtained for esterase activity in the cathodic fraction. This is not surprising as trypsin and chymotrypsin both hydrolyze esters (10). When N-cbzo-α-L-glutamyl-DL-alanine ethyl ester was employed as the substrate it was found that appreciable hydrolysis was obtained. This might be explained as follows: the enzyme first hydrolyzed the peptide bond thus releasing the alanine ethyl ester. Since the method of analysis employed measures only free amino acids, the ester group must therefore have been removed. This might be accomplished either by an enzyme or by the conditions used for assaying. The latter is probably the most important as alanine ethyl ester proved to be quite labile under the assay conditions employed. However, evidence still exists which indicates that in the presence of PRL 26 alanine ethyl ester is hydrolyzed more rapidly than the controls.

The protease-rich concentrate from PRL 26 was tested against various other substrates and no activity was demonstrated towards any of these. The dipeptides, glycylglycine, glycyl-L-proline, and serylserine were inactive. The tripeptide, diglycylglycine, was also inactive as well as N-cbzo-glycyl glycine. From these preliminary investigations it appears that PRL 26 does not contain enzymes that could be classed as dipeptidases or tripeptidases.

A considerable amout has been learned about the character of the cathodic fraction and the entire enzyme concentrate. Unfortunately very little is still known about the anodic component other than the fact that it will hydrolyze denatured hemoglobin. At the present time it would appear that the anodic fraction is a true proteinase while the cathodic component is a carboxypeptidase.

Acknowledgment

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MECHANISM OF CITRIC ACID FORMATION FROM GLUCOSE BY *ASPERGILLUS NIGER*[1]

By Ping Shu, A. Funk, and A. C. Neish

Abstract

A medium containing glucose-1-C^{14} as the sole carbon source was fermented by *Aspergillus niger* under conditions giving a steady rate of oxygen consumption and a good yield of citric acid (63%). The citric acid was isolated and degraded by chemical methods to determine the carbon-14 concentration of the methylene carbons, the tertiary carbon, the tertiary carboxyl carbon, and the primary carboxyl carbons. These were found to contain, respectively, 35.6, 21.2, 7.25, and 5.99% of the C^{14} concentration of carbon-1 of the glucose. A mathematical analysis of these data in the light of current theories on citric acid formation suggested following conclusions: (*a*) 37 – 40% of the total citric acid was formed from recycled C_4-dicarboxylic acid, (*b*) 40% of the dicarboxylic acid was formed through C_2,C_2 condensation and 60% through C_1,C_3 condensation, (*c*) 78% of the glucose was dissimilated through the Embden–Meyerhof scheme, the remainder being dissimilated through a mechanism involving carboxyl labeled pyruvic acid.

Introduction

A number of mechanisms have been suggested in the past to account for citric acid formation by *Aspergillus niger*. The significance of the role played by acetate in the citric acid synthesis has been particularly stressed. It is generally believed that a two-carbon component (presumably acetate) condenses with a four-carbon intermediate to form a six-carbon compound or citric acid. With both methyl labeled and carboxyl labeled acetate, Lewis and Weinhouse (6) demonstrated the C_2,C_4 condensation and its association with the citric acid cycle. With carboxyl labeled acetate, Bomstein and Johnson (1) arrived at the same conclusion, and under their experimental conditions a very high yield of citric acid was obtained. The C_4 intermediate may be derived either from a C_3,C_1 condensation or a C_2,C_2 condensation. With labeled carbon dioxide the incorporation of carbon dioxide in citric acid, presumably through the Wood–Werkman reaction, has been demonstrated by Foster and Carson (4), Martin, Wilson, and Burris (9), and Lewis and Weinhouse (7). The efficiency of carbon dioxide fixation was found to be rather low to account for all the C_4 compound formed during citric acid synthesis. Foster and Carson (4) observed the occurrence of C_2,C_2 condensation in *A. niger* culture besides C_1,C_3 condensation. This was further verified by Bomstein and Johnson (1) under high acid producing conditions. All these workers used preformed mycelium. Working with labeled acetate it is necessary to assume that acetate is in equilibrium with "active acetate" and

[1] *Manuscript received August 4, 1953.*
Contribution from the Prairie Regional Laboratory, National Research Council, Saskatoon, Sask. Issued as N.R.C. No. 3134.

that the latter does accumulate in substantial amounts as an intermediate during the fermentation of carbohydrates. Otherwise the conclusions derived from the experiments with labeled acetate may not necessarily be applicable to the carbohydrate fermentations. The present study deals with the mechanism of citric acid formation from glucose by a submerged culture of *A. niger* under high acid producing conditions. Glucose-1-C^{14} was used as the sole carbon source.

Methods

Culture and Fermentation

In the present study fermentation was carried out in the same manner as described by Shu and Johnson (14,16) with *A. niger* (Wis.) 72 – 4. A specially designed closed shake-flask (13) was employed. The fermenter flask simulated the conditions of cotton plugged 500 ml. Erlenmeyer flasks and contained 25 ml. of medium. A rotary shaker with 1 in. radius of motion and a speed of 230 r.p.m. was used. The carbon dioxide produced was collected in 2 *N* sodium hydroxide solution. The oxygen uptake was recorded automatically. The fermentation was carried out under a pure oxygen atmosphere at 227 mm. Hg at 30° C. and was discontinued before complete exhaustion of glucose.

Analytical Techniques

The fermentation broth was autoclaved at 125° C. for 10 min. An aliquot sample was taken for analysis. Citric acid was determined by the method of Perlman *et al.* (11). Reducing sugar was determined with Somogyi's copper reagent (18). No appreciable amount of oxalic acid was found in this fermentation.

Citric acid was recovered by the following procedure. Three volumes of absolute alcohol were added to the autoclaved culture broth to precipitate polysaccharide. The mixture of mycelium and polysaccharide was then filtered and washed several times with 75% ethanol (by volume) and finally with methanol. The washed mycelium and polysaccharide mixture was dried in a vacuum oven at 60° C. The filtrate was evaporated to a small volume and extracted with ether for three days in a continuous extractor. The ether extract was evaporated to dryness. The solid mass was reextracted with anhydrous ether for about five days. The ether extract was again evaporated to dryness. The crude citric acid was then pulverized and washed with cold anhydrous ether to remove a small amount of lipid and a trace of coloring matter. The crude citric acid was then dissolved in the least amount of methanol, and ether was added until slight turbidity appeared. The turbid suspension was then let stand at room temperature until a substantial amount of anhydrous citric acid crystallized. The precipitate was filtered, washed with anhydrous ether, and dried. The melting point of the preparation was found to be 153°–153.2° C.

The method used for the degradation of citric acid was essentially the same as that employed by Lewis and Weinhouse (6). The specific activity of the middle methylene carbon of citric acid was calculated from the specific activity of acetate and that of iodoform. All samples were converted to barium carbonate. Wet combustion of a number of samples by the method of Thorn and Shu (18) was used when necessary.

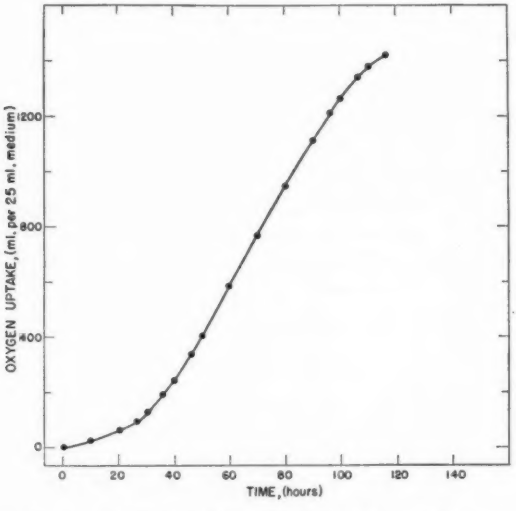
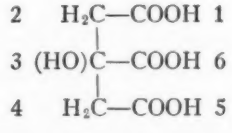


FIG. 1. Oxygen uptake in shake flask fermentation.

Results

With spore inoculum, the fermentation gave a 63% yield of anhydrous citric acid (calculated on the basis of available sugar) in about five days. The oxygen uptake curve shown in Fig. 1 indicates that after a short initial lag phase the fermentation rate was constant throughout.

For the sake of clarity, the carbons of citric acid are hereafter numbered as follows:



Most of the activity was found in the citrate (Table I). In Table II is shown that the specific activity of carbons 2, 4 was the highest, while that of the carboxyl carbons the least. There was appreciably more C^{14} in the tertiary carboxyl than in the primary carboxyls.

TABLE I

CARBON BALANCE AND THE ISOTOPE DISTRIBUTION

	Millimoles of carbon	μc. of C^{14} per gm. of carbon	Total μc. of C^{14}
Initial glucose	12.35	3.14[2]	0.465
Citric acid	7.33	3.70	0.325
Mycelium[1]	1.81	3.24	0.070
Carbon dioxide	*3.81*	*3.06*	*0.139*
Total in products	12.96	—	0.534
Per cent recovery	104.5	—	114.5

[1] *Also contains precipitated polysaccharides.*
[2] *Average of all carbons; C_1 has six times this activity.*

TABLE II

DISTRIBUTION OF CARBON-14 IN CITRIC ACID

Citrate carbon	Isolated as	Carbon-14 concentration	
		μc. per gm. carbon	% of maximum[2]
All carbons	Citric acid	3.70	19.6
1,5	Carbon dioxide	1.13	5.99
6	Carbon dioxide	1.37	7.25
2,4	Iodoform	6.71	35.6
3, $\frac{2+4}{2}$	Acetate	5.35	28.4
3	Calculated[1]	3.99	21.2
All carbons	Calculated	3.51	18.6

[1] *From iodoform and acetate.*
[2] *The maximum specific activity in carbon-1 of the glucose, i.e., 18.84 μc./gm. carbon.*

Discussion

These results may be interpreted by assuming that glucose was first split into C_3 fragments, presumably forming pyruvic acid, and then recondensed to C_6 compound (citric acid) either through the Wood–Werkman reaction or the Thunberg–Wieland condensation and followed by a C_2,C_4 condensation.

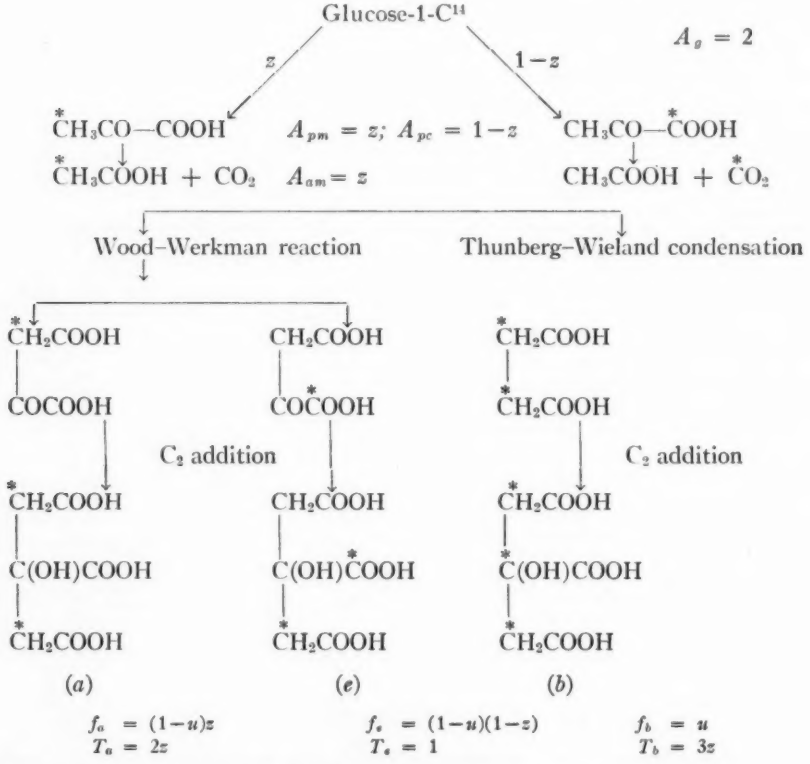
If the cleavage of glucose-1-C^{14} follows the Embden–Meyerhof scheme, methyl labeled pyruvate should be formed. If, however, the cleavage and

the pathways are similar to those found in the heterolactic organisms and *Pseudomonas saccharophila* (2, 3, 5) then carboxyl labeled pyruvate should be available for citric acid synthesis. With the assumption that the above mentioned pathways both be operative in this mold, the mechanism of citric acid synthesis by *A. niger* is systematically analyzed in the following paragraphs.

When Krebs Cycle is not in Operation

Under the conditions where the Krebs cycle is not operative, the methyl labeled pyruvic acid, through the Wood–Werkman reaction and C_2,C_4

Scheme 1. Citric acid synthesis in absence of Krebs cycle



where z = fraction of total pyruvate labeled in methyl carbon.
u = fraction of citrate initially formed through the Thunberg–Wieland condensation.
A_g, A_{pm}, A_{pc}, A_{am} are the relative specific activities of carbon-1 of glucose, methyl carbon of pyruvate, carboxyl carbon of pyruvate, and methyl carbon of acetate, respectively.
T_a, T_e, T_b are the relative activity per mole of citrate labeled as (*a*), (*e*), and (*b*), respectively.
f_a, f_e, f_b are the fractions of citrate labeled as (*a*), (*e*), and (*b*), respectively.

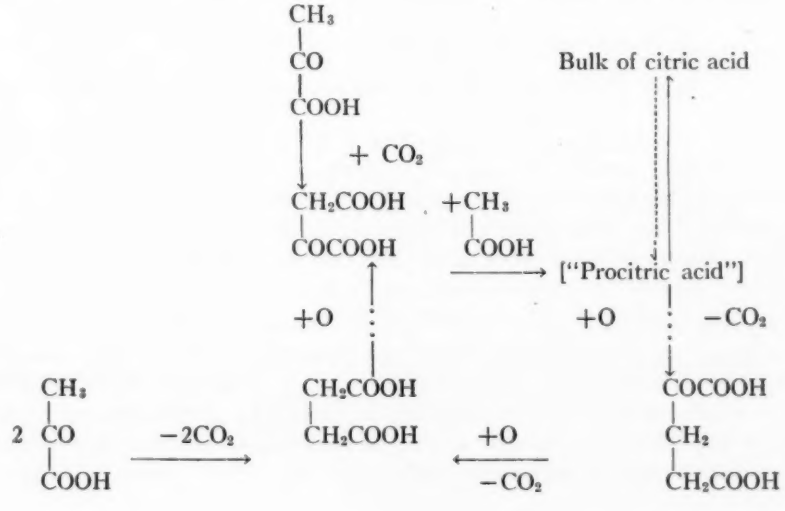
condensation, should yield citric acid labeled only at carbons 2 and 4, with their specific activities equal to that of methyl carbon of pyruvate (see Scheme 1). If, however, the C_4 compound is obtained through the Thunberg–Wieland condensation, then, carbons 2, 3, and 4 of the citric acid will be equally labeled, with specific activities equal to that of methyl carbon of pyruvate. With carboxyl labeled pyruvate, the citric acid formed will either be labeled at the tertiary carboxyl carbon or without activity at all, depending upon whether the C_4 dicarboxylic acid is formed through C_1,C_3 or C_2,C_2 condensation. For simplification of the mathematical analysis the CO_2 fixed in C_1,C_3 condensation is assumed to be inactive and a correction will be made later.

Where a mixture of carboxyl and methyl labeled pyruvic acid is formed in the fermentation, the distribution of the isotope depends upon the ratio of each type of the labeled pyruvate and also the ratio of C_1,C_3 and C_2,C_2 condensations.

When Krebs Cycle is in Operation

When a fermentation is carried out with growing cells of an aerobic organism, the Krebs cycle may be operative to some extent. In other words a certain portion of citric acid is formed from recycled C_4 dicarboxylic acid and acetate. The isotope carbon distribution in citric acid may thus be affected. The discovery of an asymmetric citric acid degrading enzyme (10, 12) has made it possible to reconsider citric acid as a member of Krebs cycle. However, by

Scheme 2. Schematic relation between Krebs cycle and citric acid synthesis



adding labeled citric acid to a culture of *A. niger* and determining the isotope carbon in carbon dioxide produced, Martin *et al.* (8) concluded that citric acid is not utilized when sucrose is present. The latter strongly indicates that the bulk of citric acid accumulated in the medium is not exchangeable with the substances in the Krebs cycle as long as carbohydrate is supplying the material for Krebs cycle operation. Thus, probably only "active citric acid" or a certain "citric acid precursor" is operative in the Krebs cycle. This existence of the "initial pool of a citric acid precursor" was suggested by Bomstein and Johnson (1). In the discussion that follows, such a precursor will be designated as "procitric acid". In an active fermentation after the initial growth phase, the amount of procitric acid, like other intermediary metabolic compounds reaches a steady state value. This value bears a constant relation to the rate of citric acid synthesis and the cycling rate of the Krebs cycle. The relation between the Krebs cycle and citric acid synthesis may be seen from the diagram in Scheme 2.

In the period when most of the citric acid was produced, the rate of citric acid production (15) and that of oxygen uptake (Fig. 1) were maintained at constant values. Thus, the amount of accumulated procitric acid as well as the fraction of procitric acid transferred to the bulk of citric acid at each cycle may be considered as constant during this period. With this consideration in mind, it is possible to estimate the fraction of citric acid produced from procitric acid subjected to various degrees of recycling. Such fractions may be expressed as:

$$[1] \qquad F_n = (1-r)r^n \qquad \text{(where } 0 < r < 1\text{)}$$

where n = number of recycling,

r = fraction of procitric acid subject to recycling,

F_n = fraction of bulk of citric acid derived from procitric acid recycled n times.

It has been established that newly incorporated acetate carbons are not removed in the first round of the cycling (19, 20, 21). Following the reactions operating in the Krebs cycle with pyruvate labeled at a known position, one can visualize the distribution of isotopic carbon in citrate derived from a procitric acid recycled a given number of times.

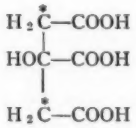
For example, with procitric acid initially formed through C_1,C_3 and C_2,C_4 condensations and using methyl labeled pyruvate as carbon source, the citric acid derived from procitric acid recycled 0, 1, 2, . . . n times may be represented by a mixture of labeled citric acid given in Table III. With equation [1] and Table III, the relative specific activity of each carbon of the citric acid thus accumulated may be estimated as follows:

TABLE III

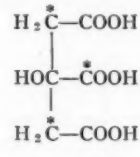
EFFECT OF RECYCLING ON THE ISOTOPE CARBON DISTRIBUTION OF CITRATE DERIVED FROM PROCITRATE INITIALLY FORMED THROUGH C_1,C_2 AND C_2,C_4 CONDENSATIONS WITH METHYL LABELED PYRUVATE AS CARBON SOURCE

Number of cycles	Mixture of labeled citric acid	Average relative specific activity at positions			
		2,4	3	6	1,5
0	I	1	0	0	0
1	II	3/4	1/2	1/2	1/4
2	II + III	7/8	3/4	1/4	1/8
3	II + III + 2IV	15/16	7/8	3/8	3/16
4	II + III + 6IV	31/32	15/16	7/16	7/32
5	II + III + 14IV	63/64	31/32	15/32	15/64
. . .					
n	II + III + $(\Sigma 2^{n-m})$ IV where $m = 2,3,4 \ldots (n-1)$, when $n \geqslant 3$	$\frac{2^{n+1}-1}{2^{n+1}}$	$\frac{2^n-1}{2^n}$	$\frac{2^{n-1}-1}{2^n}$	$\frac{2^{n-1}-1}{2^{n+1}}$

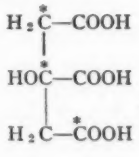
where I is



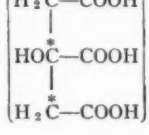
II is a mixture of equal portions of:



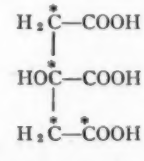
and



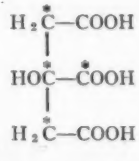
III is



IV is a mixture of equal portions of:



and



and relative specific activity = specific activity of the carbon/(half the specific activity of glucose carbon-1).

$$[2] \quad (A_{2,4})_a = z(1-r)\left[1 + \frac{3}{4}r + \frac{7}{8}r^2 + \frac{15}{16}r^3 + \ldots + \frac{2^{n+1}-1}{2^{n+1}}r^n + \ldots\right],$$

$$[3] \quad (A_3)_a = z(1-r)\left[\frac{1}{2}r + \frac{3}{4}r^2 + \frac{7}{8}r^3 + \ldots + \frac{2^n-1}{2^n}r^n + \ldots\right],$$

$$[4] \quad (A_6)_a = z(1-r)\left[\frac{1}{2}r + \frac{1}{4}r^2 + \frac{3}{8}r^3 + \ldots + \frac{2^{n-1}-1}{2^n}r^n + \ldots\right],$$

$$[5] \quad (A_{1,5})_a = z(1-r)\left[\frac{1}{4}r + \frac{1}{8}r^2 + \frac{3}{16}r^3 + \ldots + \frac{2^{n-1}-1}{2^{n+1}}r^n + \ldots\right],$$

where $A_{2,4}$, A_3, A_6, and $A_{1,5}$ are the relative specific activities of citrate carbons 2,4; 3; 6; and 1,5 respectively.

On the other hand, the isotope carbon distributions in citric acid derived from procitric acid initially formed through Thunberg–Wieland and C_2,C_4 condensations may be expressed with the following equations:

$$[6] \quad (A_{2,4})_b = z,$$

$$[7] \quad (A_3)_b = z,$$

$$[8] \quad (A_6)_b = \tfrac{1}{2}rz,$$

$$[9] \quad (A_{1,5})_b = \tfrac{1}{4}rz.$$

For the two cases described above the relations between r values and the relative specific activities of various citrate carbons (when $z = 1$) are illustrated in Figs. 2 and 3. Under no conditions are the relative specific activities of carbons 2 and 4 below 0.45 which is far above the value (0.356) obtained experimentally. This suggests that some carboxyl labeled pyruvate was also formed, and consequently, the dissimilation of glucose does not go entirely through the mechanism following the Embden–Meyerhof scheme.

The isotopic carbon distribution in citrate derived from the condensation of carboxyl labeled pyruvate (with $A_{pc} = 1 - z$) and acetate (with $A_{am} = z$) and unlabeled carbon dioxide may be calculated, in a similar manner, with the following equations:

$$[10] \quad (A_{2,4})_c = z(1-r)\left[\frac{1}{2} + \frac{3}{4}r + \frac{7}{8}r^2 + \frac{15}{16}r^3 + \ldots + \frac{2^{n+1}-1}{2^{n+1}}r^n + \ldots\right],$$

$$[11] \quad (A_3)_c = z(1-r)\left[\frac{1}{2}r + \frac{3}{4}r^2 + \frac{7}{8}r^3 + \ldots + \frac{2^n-1}{2^n}r^n + \ldots\right],$$

$$[12] \quad (A_6)_c = (1-z)(1-r) + z(1-r)\left[\frac{1}{4}r^2 + \frac{3}{8}r^3 + \ldots + \frac{2^{n-1}-1}{2^n}r^n + \ldots\right],$$

$$[13] \quad (A_{1,5})_c = z(1-r)\left[\frac{1}{8}r^2 + \frac{3}{16}r^3 + \frac{7}{32}r^4 + \ldots + \frac{2^{n-1}-1}{2^{n+1}}r^n + \ldots\right].$$

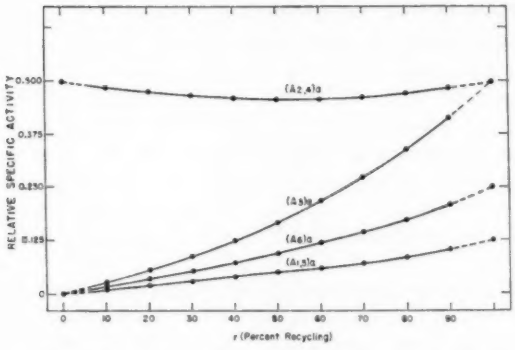


FIG. 2. Effect of recycling on the isotope distribution in citric acid when C_4 acid is initially formed through Wood–Werkman reaction.

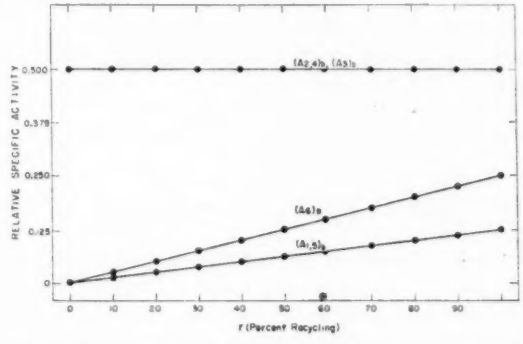


FIG. 3. Effect of recycling on the isotope distribution in citric acid when C_4 acid is initially formed through Thunberg–Wieland condensation.

Where a mixture of methyl and carboxyl labeled pyruvate was formed, and the C_4 intermediates were initially formed through both C_1,C_3 and C_2,C_2 condensations as shown in Fig. 1 ($f_a = (1-u)z$, $f_e = (1-u)(1-z)$, $f_b = u$), the distribution of the isotopic carbons in the accumulated citric acid may be represented as follows.

Relative specific activity of carbons 2,4

$$A_{2,4} = f_a(A_{2,4})_a + f_b(A_{2,4})_b + f_e(A_{2,4})_e$$

$$[14] \qquad = z(1-u)(1-r)\left[\left(\frac{1}{2} + \frac{3}{4}r + \frac{7}{8}r^2 + \frac{15}{16}r^3 + \ldots\right) + \frac{1}{2}z\right] + uz\,.$$

Relative specific activity of carbon 3

$$A_3 = f_a(A_3)_a + f_b(A_3)_b + f_e(A_3)_e$$

$$[15] \qquad = z(1-u)(1-r)\left[\frac{1}{2}r + \frac{3}{4}r^2 + \frac{7}{8}r^3 + \ldots\right] + uz\,.$$

Relative specific activity of carbon 6

$$A_6 = f_a(A_6)_a + f_b(A_6)_b + f_c(A_6)_c$$

$$[16] \quad = (1-u)(1-r)\left[(1-z)^2 + \frac{1}{2}rz^2 + z\left(\frac{1}{4}r^2 + \frac{3}{8}r^3 + \frac{7}{16}r^4 + \dots\right)\right] + \frac{1}{2}urz.$$

Relative specific activity of carbons 1,5

$$A_{1,5} = f_a(A_{1,5})_a + f_b(A_{1,5})_b + f_c(A_{1,5})_c$$

$$[17] \quad = z(1-u)(1-r)\left[\frac{1}{4}rz + \left(\frac{1}{8}r^2 + \frac{3}{16}r^3 + \frac{7}{32}r^4 + \dots\right)\right] + \frac{1}{4}urz.$$

When a set of A values are known, r, u, and z may be solved from equations [14], [15], [16], and [17]. The solution of these equations is arrived at most easily by the trial and error method. That is, one may assume a value of r, substitute it in equations [14] and [15], solve for u and z, and finally substitute these values in equation [16] to check the calculated value of A_6 against its experimental value. Thus, a number of trials may be made until the calculated value of A_6 is equal to its experimental value.

By substituting the values of $A_{2,4}$, A_3, and A_6 obtained from Table II in equations [14], [15], and [16], we have

$$A_{2,4} = z(1-u)(1-r)\left[\left(\frac{1}{2} + \frac{3}{4}r + \frac{7}{8}r^2 + \frac{15}{16}r^3 + \dots\right) + \frac{1}{2}z\right] + uz = 0.712,$$

$$A_3 = z(1-u)(1-r)\left[\frac{1}{2}r + \frac{3}{4}r^2 + \frac{7}{8}r^3 + \dots\right] + uz = 0.424,$$

$$A_6 = (1-u)(1-r)\left[(1-z)^2 + \frac{1}{2}r^2 + z\left(\frac{1}{4}r^2 + \frac{3}{8}r^3 + \frac{7}{16}r^4 + \dots\right)\right] + \frac{1}{2}urz$$
$$= 0.145.$$

Solving these equations for r, u, and z by trial and error method as described above, it was found that

$r = 0.4$; $u = 0.4$; and $z = 0.78$.

When substituting these values in equation [17], the calculated value of $A_{1,5}$ is 0.064, which is far less than the value obtained experimentally (0.12). This low calculated value of $A_{1,5}$ is expected since these equations are established on the assumption that carbon dioxide fixed through Wood–Werkman reaction is not radioactive. However this value may be corrected because the relative specific activity of the carbon dioxide fixed is known (0.322) and fixation mostly occurred through the Wood–Werkman reaction, at one of the two primary carbons (i.e. carbons 1,5) of the citrate derived from an unrecycled portion of procitrate. The true value of relative specific activity of carbons 1,5 may thus be calculated as follows:

$$[18] \quad (A_{1,5}) \text{ corrected} = (A_{1,5}) \text{ calculated} + A_{CO_2}(1-u)(1-r)/2$$

$$= 0.064 + \frac{0.324 \times 0.6 \times 0.6}{2}$$

$$= 0.122.$$

There are reports (7, 9) that a part of carbon dioxide fixed in citric acid would migrate to carbon 6. The specific activity ratio between carbons 1,5 and carbon 6 varied from 1 to 1.5. These results were obtained under the experimental conditions where the yield of citric acid was not higher than 33%. It is not known whether such migration occurs under high acid producing conditions. If the specific activity ratio between carbons 1,5 and carbon 6 is assumed to be one, then, one third of the fixed carbon dioxide should appear at tertiary carboxyl carbon and the rest at primary carboxyl carbons. Thus, equations [16] and [17] should be modified as.

$$A_6 = (1-u)(1-r)\left[(1-z)^2 + \frac{1}{2}rz^2 + z\left(\frac{1}{4}r^2 + \frac{3}{8}r^3 + \frac{7}{16}r^4 + \dots\right)\right] + \frac{1}{2}urz + A_{CO_2}(1-u)(1-r)/3 . \quad [19]$$

$$A_{1,5} = z(1-u)(1-r)\left[\frac{1}{4}rz + \frac{1}{8}r^2 + \frac{3}{16}r^3 + \frac{7}{32}r^4 + \dots\right] + \frac{1}{4}urz + A_{CO_2}(1-u)(1-r)/3 . \quad [20]$$

Substitution of the values $r = 0.37$, $u = 0.405$, and $z = 0.78$ in equations [14], [15], [19], and [20] gives

$$A_{2,4} = 0.712, \qquad A_6 = 0.158,$$
$$A_3 = 0.448, \qquad A_{1,5} = 0.10.$$

The deviations of the calculated values of A_6 and $A_{1,5}$ from the experimental values are within the range of experimental error. (Multiply these values by 50 and compare with the respective values listed under the column "percent maximum" in Table II.) If the specific activity ratio is assumed to be 1.5 the deviations will be even less.

Thus, it may be concluded that under our experimental conditions about 37–40% of the total citric acid was formed from recycled C_4 acids, 40% of the total citric acid was formed through C_2,C_2 condensation, 60% through C_1,C_3 condensation and 78% of the glucose was dissimilated through the Embden–Meyerhof scheme.

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Notes to Contributors

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